The Cannabinoid Receptor 1 gene and the Catechol-o-Methyltransferase gene in the First Episode of Psychosis

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THE CANNABINOID RECEPTOR 1 GENE

AND THE

CATECHOL-O-METHYLTRANSFERASE GENE

IN

FIRST EPISODE OF PSYCHOSIS

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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LIST OF ABBREVIATIONS:

2-AG: 2-arachidonoylglycerol

AEA: Arachidonoylethamide

ANK3: ankyrin 3

BDNF: Brain Derived Neurotrophic Factor gene

CACNA1C*:* Calcium channel, voltage-dependent, L type, alpha 1C subunit gene

CAM: Cell Adhesion Molecule

CB2: Cannabinoid Receptor 2 gene

CBD: Cannabidiol

CEQ: Cannabis Experience Questionnaire

CNR1: Cannabinoid Receptor 1 Gene

CNVs: Copy Number Variations

COMT: Catechol-O-methyltransferase

DAO: D-amino-acid oxidase gene

DAOA: D-amino acid oxidase activator gene

DLPFC: Dorsolateral Prefrontal Cortex

DRD2: Dopamine Receptor D2 gene

DSM-IV: The American Diagnostic and Statistical Manual of Mental Disorder

DSM-IV-TR: The American Diagnostic and Statistical Manual of Mental Disorder Text Revision

DZ: Dizygous

FAAH: Fatty Acid Amide Hydrolase gene

FIGS: Family Interview for Genetics Study

GAP: Genetics and Psychosis study Psychosis

GWAS: Genome Wide Association Study

ICD10 International Classification of Diseases

LD: Linkage Disequilibrium

MZ: Monozyous

NADA: N – arachidonoyl – dopamine

NAPEs: N – acyl-phosphatidylethandamines

NMDA: N-methyl-D-aspartate

NOS: Not Otherwise Specified

NRG1: Neuregulin 1 gene

NRGN*:* Neurogranin gene

PANNS: Positive and Negative Syndrome Scale

PICOS: Incident Cohort Outcome Study

PFC: Prefrontal Cortex

PLXNA2: Plexin A2 gene

PSQ: Psychosis Screening Questionnaire

RCLB: red cell lysis buffer

RGS4: Regulator of G-protein Signaling 4 gene

SSRs: Short Sequence Repeats

STRs: Short Tandem Repeats

sn-1 DAGL: *sn*-1 selective DAG lipase

SNc: Subsanta Nigra compacta

SNPs: Single Nucleotide Polymorphisms

TCF4: Transcription Factor 4 gene

THCV: Tetrahydrocannabivarin

VNTRs: Variable Number Tandem Repeats

VS: Ventral Striatum

VTA: Ventral Tegmental Area

ZDHHC8: Zinc finger binding protein gene

Δ9-THC: Δ^9 tetra-hydrocannabinol

Declaration

All my work has been guided and reviewed by my supervisors Professor John F. Powell and Professor Sir Robin Murray.

Throughout the duration of the PhD, I was responsible for the handling of DNA samples analysed in this thesis. I took care of classification, quantification, dilutions and storage.

I performed genotyping of the markers within the COMT gene using a TaqMan assay protocol and genotyping of the AATn microsatellite within the CNR1 by Fragment analysis.

During the first year of PhD I was part of the recruitment team for healthy volunteers and administered the FIGS questionnaire and the Cannabis Experience Questionnaire.

My help was also available to the group of researchers recruiting patients when and where needed.

All studies involving human subjects were approved by the South London and Maudsley National Health System Trust and Institute of Psychiatry Ethical Committee REC reference number 05/Q0706/158.

I created my own database by entering all data from paper folders; I therefore carried out all statistical analysis and writing of this work.

Genotyping of the 15 SNPs within the CNR1 gene was performed by Prevention Genetics.

Acknowledgements

I wish to thank my first supervisor Professor John F Powell and my second supervisor Professor Sir Robin Murray for their support to my work.

I would also like to thank them for giving me the opportunity to be part of the GAP group, experience that helped me improve on a scientific and personal level.

A deeply felt thank you goes to all my colleagues and principal investigators of the GAP group for the great work they have done. I feel very lucky to have been able to give my small contribution to this exceptional research group. In particular, I would like to acknowledge the group coordinator Dr Marta Di Forti for being the creator of the GAP group and for her support to all of us.

I would also like to thank all the people that have kindly given their time to fill in endless questionnaires that are so valuable to us. In particular, all clients of the SLAM services without whom this work wouldn't have been possible.

Finally, my biggest thank you goes to the few people standing by my side throughout, you know who you are, thank you!

To J and J for brightening up my life

Thank you!

Abstract

There is a general consensus in the scientific community that psychosis is a complex disorder and that its causes cannot be discovered by focusing only on one aspect. Several genes of small effect together with environmental exposure such as drug abuse are known to be important risk factors. Cannabis is the most popular recreational drug in the world and it is estimated that 40% of young people have tried cannabis sativa at least once in their life.

This study examines the role of common variations within the cannabinoid receptor 1 (CNR1) gene and the catechol-o-methyl transferase COMT gene and their interaction with cannabis use in the aetiology of psychosis.

Specifically, it analyses the main effect of 15 SNPs, namely rs10485171, rs806365, rs806366, rs12189668, rs1049353, rs806369, rs806371, rs806374, rs12195101, rs806375, rs806377, rs806378, rs2023239, rs1535355, rs6454672 and the AATn microsatellite within the CNR1 gene on psychosis and their relationship and interaction with cannabis use.

Furthermore, this study analysed the main effect of 7 SNPs, namely rs737865, rs6269, rs4633, rs4818, rs165599, rs4680 and rs2075507 within the COMT gene (4 of which form the LPS haplotype) and the LPS haplotype on psychosis and the relationship of rs4680 and the LPS haplotype with cannabis use, frequency of use and self reported experiences upon use.

Samples under investigation are part of two large studies of first episode of psychosis: the Psychosis Incident Cohort Outcome Study (PICOS) and the Genetics and Psychosis study (GAP). The GAP Study sample consisted of 2 populations, Caucasian European and Black African/Black Caribbean populations, analysed separately. The GAP Caucasian sample included 174 psychotic patients and 45 healthy subjects; the GAP Black group included 113 psychotic patients and 93 healthy individuals. The PICOS Study is based in Verona, Italy and consisted of 347 first episode psychosis patients and 307 healthy volunteers.

Results showed that rs1049353 and rs806378 within the CNR1 gene were associated with psychosis in the GAP Caucasian sample (adjusted p-value=0.03) and (adjusted p-value=0.05).

Subjects with 2 copies of the LPS haplotype experienced more perception abnormalities. This however did not retain significance after Bonferroni correction for multiple testing (adjusted p-value=0.08 and p-value= 0.16 respectively).
Subjects using cannabis more than 3 times per week were more likely to respond with anxiety and or paranoia to the drug (adjusted p-value=0.004).

Subjects using cannabis more than 3 times per week were also more likely to have a pleasurable experience upon cannabis consumption. This did not however retain significance after multiple testing correction (adjusted p-value=0.2).
Associations observed were not disease group or ethnicity dependent.
No other association was found to be significant before or after Bonferroni correction for multiple testing.

Because of the few samples available, this study is underpowered and in light of many other limitations it is unable to detect any true association. Results though should be interpreted as possible indication of involvement of the CNR1 and the COMT gene in the aetiology of psychosis.

CHAPTER 1

INTRODUCTION

PSYCHOSIS: CLINICAL PRESENTATION

1.1 Psychosis

Psychosis is a term derived from the Greek ψυχή – psyche – meaning 'soul' and the suffix ωσις – osis – meaning 'abnormal condition'. The American Diagnostic and Statistical Manual of Mental Disorder (DSM-IV) (American Psychiatric Association, 1994) includes in the psychosis spectrum, nine formal psychotic disorders: schizophrenia, schizoaffective disorder, schizophreniform disorder, brief psychotic disorder, delusional disorder, shared psychotic disorder, psychosis not otherwise specified (psychosis NOS) and psychosis caused by medical condition (organic psychosis). Psychotic symptoms may be also present in major depressive disorder and bipolar disorder. They are grouped together to facilitate the differential diagnosis of disorders that include psychotic symptoms with their presentation, like for example, Alzheimer's dementia or substance induced delirium.

At present, psychosis is used as an 'umbrella' term used to identify a group of heterogeneous disorders. They are all characterised by behavioural and perceptual disturbances, severe impairment in function at social and personal level preventing the affected individual from performing everyday tasks.

The disorder we now call Schizophrenia was first described in 1896 by Emil Kraepelin, a German professor of psychiatry. It was characterised by an early an age of onset, deteriorating clinical course and poor outcome, symptoms of hallucinations and delusions were also present. Kraepelin named the disorder 'Dementia praecox' which was different from manic-depressive insanity in familiarity, temperament and onset. Kraepelin particularly stressed the importance of family history, cognitive impairment and thought it was of organic origin (Lehmann 1980; Hoenig 1983). He described a mixture of symptoms of motor deficits, delusions, hallucinations, avolition and social isolation. This was therefore the first description of a psychotic disorder with a genetic basis.

The term schizophrenia was then introduced in 1908 by Eugen Bleuler, a psychiatrist from Switzerland. The term comes from the Greek 'schizo' – split – and 'phrene' – mind- to emphasise the fragmented thinking characteristic of affected individuals. Bleuler described schizophrenia with the 'four A's': loosening of Association, Autism, Ambivalence and blunted Affect; 'thought disorder' was therefore the main symptom at the centre of his classification.

According to the DSM-IV (American Psychiatric Association, 1994), schizophrenia presents with a mixture of positive and negative symptoms that have to be present for a consistent proportion of time during 1 month period with some signs persisting for at least 6 months time.

Both the DSM-IV and the International Classification of Diseases 10th edition (ICD-10) take the first three sub-types from the original Kraepelian classification.

According to the DSM-IV there are six criteria to diagnose schizophrenia:

Criterion A – Characteristic symptoms (delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behaviour): two symptoms have to be present for at least the duration of 1 month. However, only 1 characteristic has to be present if delusions are bizarre, or hallucinations consist of one or more voices conversing together, or if a voice runs a commentary about the behaviour of the person.

Criterion B –Social/occupational dysfunction: one or more areas of self care, work or interpersonal relationships are below the level it was prior the onset of the disturbance.

Criterion C –Duration: signs must persist for at least six months with 1 month of symptoms from criterion A.

Criterion D –Schizoaffective and Mood disorder exclusions: schizoaffective disorder and mood disorder with psychotic features must be ruled out.

Criterion E –Substance/general medical condition exclusion

Criterion F –Relationship to a pervasive developmental disorder (PDD): the diagnosis of schizophrenia can be made in the presence of PDD only if delusions and hallucinations are predominant for at least the duration of 1 month.

The DSM-IV TR in the section of Schizophrenia and other psychotic Disorders also includes: Schizophrenia, Schizophreniform Disorder, Schizoaffective Disorder, Delusional Disorder, Brief Psychotic Disorder, Shared Psychotic Disorder, Psychotic Disorder Due to a General Medical Condition, Substance-Induced Psychotic Disorder, and Psychotic Disorder Not Otherwise Specified.

Schizoaffective Disorder

Schizoaffective disorder is characterised by mood symptoms as well as psychotic symptoms. To meet the criteria for diagnosis, together with the symptoms that meet criterion A for schizophrenia, there have to be present a major depressive episode, a manic episode or a mixed episode or psychotic symptoms for at least two weeks in absence of mood symptoms.

Brief psychotic episode

The diagnosis of brief psychotic disorder is made when psychotic symptoms like delusions, hallucinations, disorganised speech and grossly or catatonic behaviour are present for at least a day but less than a month.

Delusional disorder

Delusional disorder is mainly characterised by the presence of delusions for at least 1 month duration. Tactile or olfactory hallucinations can also be present if part of the delusion. Functioning is maintained apart from the impact of the delusion itself and mood episodes, if present are usually brief. The disorder is not due to the effect of abuse of drugs.

Schizophreniform Disorder

Schizophreniform disorder can be characterised by confusion or perplexity at the height of the psychotic episode but the subject can function properly socially and occupationally during the premorbid phase. Blunted or flat affect are absent. For the diagnosis of schizophreniform disorder, criteria A, D and E for schizophrenia are met. The episode should last at least 1 month but less than 6 months.

Shared psychotic disorder

Shared psychotic disorder is characterised by the development of psychosis by an individual in a close relationship with another with established delusions. It has also been called ‘Folie a deux’ and it requires the absence of psychotic disorder prior to the onset of delusions.

1.2 Psychotic symptoms

Psychotic symptoms are divided into positive and negative symptoms, where positive indicates an excess or distortion of normal functioning and negative indicates a reduction or total loss of normal function. According to the DSM-IV TR (American Psychiatric Association, 1994) positive symptoms include: hallucinations, delusions, disorganised speech, grossly disorganised or catatonic behaviour whereas negative symptoms include: alogia, affective flattening and avolition.

Positive symptoms

Hallucinations

The term hallucination comes from the Latin word 'alucinari' meaning 'to wonder in mind' and was introduced in 1837 by Esquirol.

Hallucinations can be defined as perceptions in conscious state in absence of the stimuli which have qualities of real perception. Hallucinations are usually vivid and located in external object space and can occur in many modalities: visual, auditory, olfactory and gustatory. The most common type by far is auditory hallucinations which are perceived as familiar or unfamiliar voices clearly distinct from a person own thoughts. Voices are usually nasty and can be threatening for the individual; they can be in the form of derogatory comments or commands. Very characteristic of schizophrenia are hallucinations consisting of two or more voices running a commentary on the person's behaviour, if this is present, it is enough to meet the criteria for the diagnosis of schizophrenia.

Visual hallucinations: phenomena of seeing things that are not present and do not reflect reality.

Olfactory hallucinations: phenomena of smelling odours that are not derived from any physical stimuli. Odours are often unpleasant.
Tactile hallucinations: phenomena of a sensation of tactile stimuli with the absence of concrete stimuli. Tactile hallucinations are much less frequent than auditory hallucinations.
Gustatory hallucinations: involve the sense of taste. The individual may report simple tastes like sweet or salty and the phenomena are usually associated with olfactory hallucinations.

Delusions

Delusion can be defined as false beliefs. They are usually not consistent with the individual's religious or cultural background. Delusions are very challenging because they are very difficult to eradicate. They are usually not open for discussion and can also be much elaborated. Types of delusions can be divided into: delusions of control, delusions of jealousy, nihilistic delusions, delusions of reference, delusions of grandiosity, persecutory delusions, somatic delusions, erotomanic delusions and mixed type delusions.

Delusions of control: false belief where the individual is certain somebody else, some external force or a group of people, is controlling their actions or thoughts.

Delusions of jealousy: false belief of the individual's partner being unfaithful. The individual may gather evidence and confront the partner about non-existent facts.

Nihilistic delusions: false beliefs that focuses on the non existence of self or part of self.

Delusions of reference: false beliefs that insignificant remarks or insignificant words have a personal significance to the affected individual. The individual may take as personal and very meaningful newspapers heading or words overheard from people passing by.

Delusions of grandiosity: the delusion is often well articulated, the individual is strongly convinced to have an occupation or a role of prestige and does not show any sign of insight.

Persecutory delusions: belief of an individual that he or she is being persecuted. The subject often reports the feeling of being spied on or followed and as a result can be dangerous.

Somatic delusions: false belief concerning body part or loss of body functions.

Erotomanic delusions: false belief of an affected individual that another person, often a stranger, is in love with him/her.

Mixed type delusion: all kind of delusions that do not specifically fall into any of the above categories.

Disorganised speech

Disorganised speech is diagnosed when speech and behaviour are considerably disorganised or jumbled. The individual may continue to ask the same question or jump from a subject to another without giving proper notice. When this occurs, there can be the impression of an overflow of thoughts going on in the individual's mind. The subject may also attempt to express feelings or to tell a story without managing to keep the point, the conversation is therefore perceived by others as incoherent or illogical.

Negative symptoms

Alogia

The term alogia comes from the Greek words 'α' meaning without and 'λόγος' meaning speech. Alogia is used to indicate lack of speech or poverty of speech, or more often, a lack of unprompted speech seen in normal situations.

Affective flattening

Affective flattening can be observed in individuals presenting with psychotic symptoms and consists of a blunt response to emotions. The individual may present with total absence of emotional response or with a restricted response.

Avolition

Symptoms of avolition are characterised by lack of drive, desire and goal directed behaviour. It can sometimes be mistaken for disinterest but it differs in severity. The individual may become disinterested in going out with friends or carrying out everyday activities; the result can be a total absence of any social interaction or activity. Usually, negative symptoms tend to cluster and present together with variable severity and in combination with some positive symptoms.

CHAPTER 2

INTRODUCTION

GENETICS AND PSYCHOSIS

2.1 Early genetic findings

Psychosis, as mentioned earlier, is an 'umbrella' term used to indicate several disorders with similar features. The incidence of schizophrenia is thought to be between 0.5 and 1% of the general population (Jablesky A. et al., 1992).

Since Emil Kraepelin coined the term 'Dementia Precox' referring to today's diagnosis of schizophrenia, there has been significant amount of research done aiming to understand the aetiology of psychosis. The very first evidences of a possible genetic basis for psychosis date back to 1916. Ernst Rudin performed the first family study on what was then called 'Dementia precox' and found that the rate of the disease was significantly higher among siblings of probands. More recently, family studies as well as molecular studies highlight the likely presence of shared genetic risk factors between schizophrenia and bipolar disorders. Familiarity of schizophrenia has been shown to be between 41% and 87% (Cardno et al, 1999; Kendler et al, 1983) familiarity of bipolar disorder to be between 73% and 87% (Cardno et al, 1999; Kendler et al, 1995, McGuffin et al, 2003). Moreover, the risk of developing bipolar disorder is also increased by having a relative with schizophrenia (Kendler et al, 1993a,b,c; Maier et al., 1993, 2002). These findings have been supported by a recent study in 2009 by Lichtenstein et al, where the authors linked multi-generation register and hospital discharge register identifying more than 2 million nuclear families between 1973 and 2004. The results indicate an overall increase in relative risk for both schizophrenia and bipolar disorder. These findings are consistent with several genetic and neurobiological findings further discussed in this thesis.

2.1.1 Twin and Family Studies

Family studies have shown familiarity of the disorder giving us insight into possible genetic cause of psychiatric disorders. However, one cannot be sure whether such findings are due to genetic sharing or to environment sharing. Twin studies are very valuable in the search for genetic basis of psychosis since identical twins are thought to share 100% of their DNA. The first twin study was carried in Munich by Luxenberger in 1928. Subsequently Kendler in 1983 carried out a systematic review of all twin studies to date and concluded that indeed there were higher concordance rates

between MZ twins (concordance rate between 41% and 87%) highlighting the genetic influence in psychotic disorders.

In order to explore the role of environmental sharing, adoption studies are design to allow us to control for post-natal family environment. Adoption studies can follow three different designs:

1. Adoptees studies where adopted away offspring of affected parents are compared with adopted away offspring of non affected parents;

2. Adoptees family study where the adoptees biological parents are compared to adoptees adoptive parents for risk of disease and;

3. Cross-fostering studies where offspring of affected individuals are reared by healthy adoptive parents are compared with children born from non affected individuals and reared in a family where a parent develops the disease.

First studies using adoptees study design were performed by Heston in 1966 and Rosenthal et al. in 1971. They both concluded that there was significant higher risk of schizophrenia in adoptees born from an affected parent. Lately a Finnish study results showed an increase morbid risk (8.1%) in adopted away children of affected mothers compared with children of non affected mother where the morbid risk was 2.3%. (Tienari et al., 2000).

All these studies suggest that schizophrenia and in fact psychotic disorders cluster in families and that genetic liability plays an important role. However, as suggested by Tienari et al in 2004, high risk adopted away children do experience more adverse life events than children reared with healthy parents, putting forward the argument for gene and environment interaction.

2.1.2 Linkage Studies

One of the main classical methods of detecting chromosomal regions associated with schizophrenia has been linkage analysis. The principle of genetic linkage is that given one or more alleles on different loci which are closely localised on the same chromosome, tend to be co-transmitted as there is a low probability of recombination between them.

The Schizophrenia Research Forum quotes that to date 32 major linkage studies have been performed in schizophrenia spectrum disorders and also four meta- linkage analyses. Results from a meta-analysis performed by Badner and Gershon in 2002 identified three main regions: chromosome 8p, 13p and 22q.

One of the most interesting results of the linkage studies is the discovery of region 22q. A deletion on 22q11 causes a disorder called Velo-Cardio-Facial syndrome. Affected individuals are at higher

risk of developing schizophrenia compared to the general population. In a study by Murphy et al., in 1999, rates of schizophrenia among 50 adult males with Velo-Cardio-Facial syndrome were estimated to be 24%, therefore, putting this population at very high risk. Although this deletion does not account for the majority of cases of schizophrenia, certainly gives some insight to a genetic loci implicated in the disease. Interestingly, among many the genes lying on the 22q area of the chromosome, the COMT gene analysed in this study is one of them. The COMT gene is particularly interesting as it plays a key role in the catabolism of dopamine, and of course dopamine is implicated in the pathogenesis of schizophrenia (Howes and Kapur, 2010). Linkage studies nowadays can be carried out in much larger scale with genome wide linkage studies. Suarez et al., (2006) conducted a study in 409 European and American ancestry families and found areas in chromosome 8p23.3-p21.2 in schizophrenia and areas in 11p13.1-q14.1 in both schizophrenia and bipolar disorder.

Even more recently, the work of Byerley et al., (2011) implicated chromosome 16p in bipolar disorder. Vassos et al., (2012) suggested strong association at the 3p21.1 locus for bipolar disorder. However, linkage studies have not been successful in consistently identifying loci in schizophrenia. Some of the reasons of this lack of consistency might be related to the facts that Schizophrenia is now considered a complex disorder consequent upon many small genes of small effect rather than few genes of major effect. In addition, it may also be due to the heterogeneity of the sample studied given the fact that schizophrenia can be considered a spectrum rather than a single disorder.

2.3 Association Studies

Association studies examine whether genetic variants are associated with a disease trait. Alleles or genotypes frequencies are analysed in both diseased and control populations. Although study designs may vary, there are three main ones:

1. Cohort study design using a sample to be followed up (prospective study);

2. Family based design using families with one or multiple affected individual or trios, usually proband and both parents;

3. case-control design using the same number of case and matched controls (some studies may require larger number of controls in order to reach enough statistical power).

Genetic markers can be of different types with the most commonly analysed being Single Nucleotide Polymorphisms (SNPs). SNPs are point mutations occurring on a single nucleotide and are frequent within the general population with allele frequency of >1%. Association studies explore the difference in allele frequency between cases and controls with the hypothesis of: if the studied allele is involved in the pathophysiology of the disorder, it should be over expressed in the

cases population. Other types of markers analysed in association studies can be rarer point mutations (freq. <1% in general population) or Copy Number Variations. Association studies in psychosis as in other complex disorders have been proven to be difficult because of multi-factorial or multiple genes involved and also their interaction with environmental factors.

From association studies it is possible to detect both directly and indirectly associated variants, where the first is directly interacting with the trait and the second in Linkage Disequilibrium with an untyped causative variant. Linkage Disequilibrium refers to the non random association of alleles at two or more loci. In a recent review it was shown that there are over 1400 association studies have been carried out in schizophrenia covering around 6550 polymorphisms within 761 genes (Hon-Cheong et al., 2009). Molecular genetics techniques have evolved exponentially in the past few years allowing the analysis of many variants at the same time in much larger samples. This has led to the increase in positive as well as negative associations. During the years, the research for genetic markers has focused on candidate genes, mainly important genes in the regulation of implicated neurotransmitters or genes located on or in the proximity of an area that returned significant results after linkage analysis (The Schizophrenia Research Forum)

With the candidate gene approach, genes within the dopaminergic system have widely been investigated with the dopaminergic receptor genes DRD2 and DRD3 being among the favoured candidates (Ariami et al., 1994) (Kaneshima et al., 1997) (Dubertret et al. 2001) (Crocq et al., 1992) (William et al., 1998). However, many associations failed to be replicated (Harano et al, 1997) (Nanko et al., 1994) (Sanders et al., 1993) and other findings seem not to agree with previous studies (Elvidge et al., 2001).

The DAO and DAOA genes situated on chromosome 13q have been also extensively studied in schizophrenia. Both showed association with schizophrenia (Sullivan et al., 2000) (Detera-Wadleigh et al., 2006) with DAOA also implicated in Bipolar Disorder (Prichard et al., 1992) (Williams et al., 2006).

In addition, another widely investigated dopaminergic gene has been COMT gene. As mentioned before, COMT is situated on the long arm of chromosome 22 at 22q11 position. The same region returned positive results in several linkage analysis and meta-analysis (Pulver et al., 1994) (Badner and Gershon, 2002). A functional polymorphism (Val158Met) situated in the COMT gene has been extensively studied and a haplotype within the COMT gene has been found significant both in schizophrenia (Shifman et al., 2002) and bipolar disorder (Shifman et al., 2004). Further details on the COMT gene and its implications in schizophrenia are given in chapter 3 of this thesis.

Another gene that has gained attention as a candidate gene in schizophrenia is RGS4. It is situated on the long arm of chromosome 1 and has been found to be expressed less in brains of patients with

schizophrenia (Mirnics et al., 2001). In addition, RGS4 has been associated with schizophrenia by several studies, with stronger evidences coming from a haplotype at the 5' end of the gene (Chowdari et al., 2002).

Association studies are based on the association of a specific phenotype or marker for the disease with the candidate gene with a putative role in that disease trait. These studies should be more powerful than linkage studies as it directly tests the disease marker. However, although many of the described association studies have shown positive results, they are not consistently replicated. It has been argued to be related to small sample size to detect a genetic effect and therefore, GWAS approach has reached more popularity in genetic research.

2.4 Genome Wide Association Studies

The aim of GWAS is to genotype very large numbers of SNPs genome wide in a case-control design analysis. GWAS represent the newest method that offers a hypothesis-free analysis as it analyses the whole genome and have been crucial to the identification of genetic variations associated with many diseases including for example Type II Diabetes, Crohn's Disease and breast cancer. GWAs commonly used panels include up to 1 million SNPs plus markers in linkage disequilibrium (non-random associations of SNPs). In a GWAS, all subjects included in the group of cases should meet lifetime criteria for the disease and controls should be drawn from the same population and should never have met the criteria for the disease (Corvin et al., 2010). GWAS are used to test individuals for point mutation within the DNA, but they are also an important tool to detect Copy Number Variations (CNVs), DNA duplications and deletions (Hosak et al., 2012).

In association studies such as GWAS, the strength or effect size is usually measured with Odds Ratio (OR) as proportion of variance explained for "quantitative trait" such as brain volume or for "discrete trait" such as diagnosis. In this way, the SNPs identified are believed to mark a region of the human genome that increases the risk for the illness (Pearson and Manolio, 2008). The advantages of the GWAS over candidate gene association studies lies in the possibility of looking at the whole genome at once rather one or few genetic regions. Moreover, the quality control that these studies undergo is much more restrictive than candidate gene approach. For example population stratification can be adjusted using information from the genetic markers that enable the fine estimation of ethnic stratification. In this way, ancestral genetic heterogeneity can be better controlled for. Despite these advantages, GWAS studies also carry some disadvantages, for instance, in order to reach Genome Wide significant, a multiple correction is needed, that is to correct for the number of SNPs analysed. Significant p-values in GWAS are therefore smaller than ordinary association studies and less than 10^{-8}. Association can be replicated in an independent and larger sample which is usually more difficult to attain (Pearson and Manolio, 2008) or explored further with meta-analysis.

The first GWAS was published in 2005 by Klein et al. for macular degeneration disease, and to date there have been several systematic Genome Wide Studies (GWAS) in schizophrenia. In the last years there has been an increase in findings of susceptibility loci in schizophrenia thanks to the newest techniques and collaborations like the Schizophrenia Consortium. More than 70 independently associated alleles were found with GWAS studies, all of which of a small effect size which together they may explain less than 5% of the genetic variance in schizophrenia (Corvin A., 2013).

GWAS performed in schizophrenia supports the idea of a polygenic disorder with a complex biological aetiology; therefore, several genes of very small effect may each contribute to the heterogeneous phenotype observed clinically. By increasing the sample size, several genes implicated in neurodevelopment, immunology, neuroplasticity and neuroendocrinology are emerging, following the trend previously observed in other diseases like Type 2 diabetes and Crohn disease (Corvin A., 2013). However, as noted by Corvin, some variants will have such a small effect, that could go undetected. One of the genes emerging from the GWAS studies is the ZNF804A (O'Donovan et al., 2008) (Shi et al., 2009) which has also been associated with neural activation during memory tasks in healthy volunteers by Hashimoto et al. in 2010. Hashimoto et al. (2010) also demonstrated poorer performance on verbal and visual memory and delayed recall in patients with schizophrenia ($p<0.001$). Other genes found significant in GWAS, like CACNA1C, TCF4 and NRGN (Stefannson et al., 2009) (Bergen at al., 2012) (ISGC&WT, 2012) (Arberg et al., 2013) (Smoller et al., 2013) are genes involved in ion channel and synaptic function and target of specific miRNA (O'Donovan et al., 2012).

Neurodevelopmental genes have also been implicated in other disorders such Intellectual Disability and Autism with the latest findings coming from the GWAS performed by Smoller et al., in 2013. Several overlapping genes have been found, thus suggesting the possible implication of factors important for neuroplasticity and neurodevelopment, like synapse maturation and receptor abnormalities (Hosak et al., 2012). Furthermore, the PRODH gene, also reported significantly associated by GWAS (Alkelai et al., 2011) in pre-frontal cortex and striatum connectivity and function in a family based study of schizophrenia. Moreover, it has also been described in the pathophysiology of schizophrenia with a risk haplotype associated with decreased striatal volume (Li et al., 2008). It was also found that the striatal frontal functional connectivity was increased (Li et al., 2008).

In addition, genes like the HLA gene, significant in GWAS (Stefannson et al., 2009) (Shi et al., 2009) (Purcell et al., 2009) (Bergen et al., 2012) (ISGC&WT, 2012) has given rise to an immunological hypothesis of schizophrenia.

It is important to note that findings of GWAS, although highlighting the tip of the iceberg, give us important clues on the biology underlying the disorder. It is quite striking the fact that previously associated loci by association studies in candidate genes are not being seen significant in GWAS. This could be due to the fact that previous effect sizes might have been over estimated in these studies. With the current hypothesis of schizophrenia, like other complex disorders, being a polygenic syndromic disorder, we know of the presence of many variants of very small effect each. As mentioned earlier, some variant may have such a small effect that would be undetected by statistical analysis; we therefore understand the importance of a very large sample size, which is achieved mainly through collaboration in GWAS. This is one of the strength points of this type of association studies. It is perhaps only now, despite of many years in search for pathogenic variants, that the genetic structure of schizophrenia is being unrevealed; one of the next steps will surely be sequencing of the whole exome in a large sample size. This will not only underpin common variants of small effect, but also larger rarer variants of a much bigger effect, thus helping resolving a step further a difficult puzzle.

Table 2.1 Latest findings from GWAS performed in Schizophrenia

(Data taken and readapted from http://www.genome.gov/gwastudies)

STUDY	POPULATION	PLATFORM	NUMBER OF SNPs	NUMBER OF CASES	NUMBER OF CONTROLS	ASSOCIATED GENES	BEST EFFECT SIZE
Lencz et al. 2007	USA	Selected cSNPs	25494	178	144	CSF2RA IL3A	Not stated
Kirov et al., 2008	Bulgaria	Illumina HumanHap (550K)	433680	574	605 1148 parents of cases	CCDC60	Not stated
Shifman et al., 2008	Israel	Affymetrix	510552	660	2771	RELN	1.58 (1.31-1.89)
Sullivan et al., 2008		Affymetrix		738	733	AGBL1 ACSM1 BUCS1	6.01
Walsh et al., 2008		Illumina		150	268	NRG1	
O'Donovan et al., 2008		Affymetrix		479	2937	ZNF804A 2 Intergenic regions	1.12
Stefansson et al., 2009	Europe (SGENE-Plus)	Illumina HumanHap	314868	2663	13498	MHC PRSS16 NOTCH4 NRGN TCF4 VRK2 SLCO6A1	1.23
Shi et al., 2009	Europe African/American	Affimetrix		2681 (Europe) 1286 (African American)	2653 (Europe) 973 (African American)	SLC17A1 SLC17A3 BTN3A2 BTN3A1 HIST1H2AG HIST1H2BJ PRSS16 POM121L2 ZNF184 HLA-DQA1	1.28

Purcell et al., 2009	Europe	Affimetrix		3322	3587	MHC TCF4 FXR1 PTBP2	1.44
Athanasiu et al., 2010	Norway, Europe (SGENE-Plus)	Affimetrix 6.0	572888	201	305	PLAA ACSM1 ANK3	1.16
Ma et al., 2011	Chinese Ancestry	Illumina		98	60	MRSA	
Alkelai et al, 2011	Jewish Israeli	Illumina		331 family members		ATP5SL CEACAM21	
Ripke et al., 2011	Europe	Affymetrix &Illumina		9394	12462	TRIM26 MIR137 CNNM2 CSMD1 CCDC68 NT5C2 PCGEM1 ITIH3 ITIH4	1.22
Yue at al., 2011	Chinese	Illumina		746	1599	NKAPL TSPAN18	1.29 (1.23-1.36)
Shi et al., 2011	Chinese	Affymetrix		3750	6468	LSM1 WHSC1L1 BRP44 DCAF6	1.23 (1.15-1.32)
Liou et al., 2012	Han Chinese	Affymetrix		522	806	NFKB1 SLAMF1	1.45 (1.26-1.66)
Wang et al., 2012	Europe	Affymetrix		835	2694	PKNOX2 KCNJ2 JRKL INSIG1 GJA4	1.89

Bergen et al., 2012	Europe	Affymetrix		2111	2535	MHC NT5C2 CNNM2 MAD1L1 TCF4 CACNA1C	1.24
Irish Schizophrenia Genomics Consortium & the Wellcome Trust Case Control Consortium	Europe	Affymetrix		1606	1794	MHC TRIM26 CACNA1I	1.22 (1.12-1.39)
Levinson et al., 2012	Illumina	Europe		1218	990	EXOC2 PPFIA2 TMTC1	1.97
Betcheva et al., 2012	Illumina	Europe		188	376	HHAT	2.63 (1.89-3.66)
Fanous et al., 2012	Europe	Affymetrix		2454		CTDP1 ADAMTS6 CXCL12 PCDH20 RORA	1.536 unit decrease
Xu et al., 2013	Europe	Affymetrix		1774	2726	BCL9 C9orf5 ST3Gal1	1.6
Bolgrum et al., 2013	Europe	Illumina		888	882	RUNDC2A CDH13 ARNTL	1.342
Aberg et al., 2013	Europe			11185	10768	TCF4 NOTCH4 POM121L2 SEC16B	1.6
Smoller et al., 2013	Europe			9379	27888	ITH3 MHC CCDC68 ANK3	1.1 (1.07-1.12)

2.5 Copy Number Variation Studies

Copy Number Variations (CNVs) are polymorphic structural variations of a segment of DNA of at least 1kb in size. Variation of less than 500K in human DNA in copy numbers, are far more common than initially thought, as demonstrated by Sebat et al. and Lafrate et al., in 2004. Although estimated to be of approximately more than 1000 in the genome, and therefore far less in number compared to SNPs, their estimated contribution to the total variation in the human genome is thought to be very similar, due to their size (Malhotra and Sebat., 2012). This is to say that although CNVs occurrence is lower and their rate of structural mutation is also low, they can span tens of thousands of kilobases per site and therefore have a larger functional impact (Melhotra and Sebat., 2012). CNVs have revolutionised the way we understand the genetic architecture of complex disorders like Autism Spectrum Disorders, Intellectual Disabilities, Bipolar Disorder and Schizophrenia. Sebat et al., in 2009 demonstrated a significant role of rare (<1%) and large (>100kb) in schizophrenia and several studies observed a 1 to 3 folds enrichment in cases versus controls (Walsh et al., 2008) (ISC, 2008) (Kirov et al., 2009) and in "sporadic" cases versus "familial" cases (Xu et al., 2008). These findings were however not replicated by subsequent studies, although a higher rate (5%) of de novo CNVs was observed in patients versus controls. (Kirov et al., 2012) (Malhotra et al., 2011).

Several regions of the genome have been implicated in schizophrenia, with the most common being in genes related to neurodevelopment (Walsh et al., 2008), in support of the neurodevelopmental hypothesis of Schizophrenia. Kirov et al., in 2012 demonstrated in a recent experiment that components of postsynaptic density like the neuronal activity-regulated cytoskeleton-associated protein postsynaptic signalling complex and the NMDAR receptor were significantly more present in de novo CNVs (Kirov et al., 2012).

The two models that are coming out in view of the recent findings are the Common Variants Common Disease model, where multiple variants at multiple loci play small and different role in the pathophysiology of the disorder (causative, modulator, protector, and mediator) and; the Rare Variant Common Disease model, where larger rarer structural variants confer a higher risk for the disease. However, these two models are not mutually exclusive as it is plausible that both models could act together within the same disorder. Schizophrenia is a syndromic disorder characterised by three main clusters of symptoms, such as positive, negative and cognitive symptoms, as discussed in Van Os and Kapur, 2009. Therefore, it is thought that different structural variations or common polymorphisms might produce different symptomatology on a continuous scale giving rise to the development of a particular symptom, the variety in intensity of symptoms or the duration particular symptoms in psychotic illnesses. Furthermore, the same structural variation may be found at a lower rate in the control population, as noted by Malhotra and Sebat in 2012 highlighting the

issue of possible modulators like epigenetic factors or the role of the environment for the development of the disorder.

2.6 Gene x Environment Interaction Studies

In order to understand complex disorders such as schizophrenia, genetic information should be analyzed together with the putative influential environmental factors. This thought has led to a prolific area of research in schizophrenia with several interesting findings on the role of the environment in the development of the illness.

Studies looking at the role of environmental factors have described that early environmental insults such as winter birth can influence the development of schizophrenia. In addition, obstetric complications have been widely studied and it is now accepted that they confer an increase risk of schizophrenic disease. Cannon, Jones and Murray in 2002 conducted a systematic meta-analysis of all paper published on the subject and found that obstetrical complications could be grouped in three separate groups: 1. Complications during pregnancy; 2. abnormal foetal growth and 3. complications during delivery. All categories conferred higher risk of the disease with effect sizes of less than two (Cannon, Jones and Murray, 2002). Nutritional deficiency during pregnancy has been associated with risk of schizophrenia and in patients with schizophrenia has been shown to be associated with decreased intracranial volume and other brain abnormalities (Hullshoff et al., 2000).

Another well reported environmental factor to influence the development of psychosis is "stressful life events" which started to gain recognition as a risk factor for psychosis after the study by Brown and Birley in 1968. Since then, an increase in stressful life events in the months prior to the onset of a psychotic episode have been reported (Bebbington et al., 1993). Interestingly, stress has also been associated with relapses in psychosis (Ventura et al., 1989). Despite these promising results where there seems to be a correlation between stressful life events and the risk of developing psychosis, it is very important to further explore whether stress has a causal relationship with psychosis or if it is a consequence of the disorder.

Another field of investigation into environmental predisposing factors is investigating in traumatic experiences that happen early in childhood, in particular childhood physical and sexual abuse or loss of a parent. Many studies have shown an association with exposure to trauma early in life and predisposition to psychiatric disorders (Nemeroff, 2004, Stein et al., 1996). For instance, early childhood traumatic experiences have been reported to occur in excess in psychosis and also in anxiety disorders (Stein et al., 1996).

Another environmental factor is urbanicity which has also long been considered a risk factor for psychosis. The first studies linking urban cities to increased risk of psychosis date back to 1956 by

Hare. In a meta-analysis McGrath et al., in 2004 showed that the risk of Schizophrenia is higher in cities rather than in mixed or rural areas. The incidence of psychotic disorders in the UK can be considerate higher in certain areas like the South-East London (Kirkbride et al., 2006). In a meta-analysis, Jim Van Os and colleagues analysed findings from 10 separate studies. They found that the rate of schizophrenia in urban cities can be considered doubled compared to rural areas; the statistical analysis was corrected for several confounders like gender, age, education and drug abuse (Van Os et al., 2005). These finding seem to be robust across Countries and Cultures.

Finally, substance misuse is another environmental factor that has captured a lot of attention for the schizophrenia research in particular gene environment interaction. It is estimated that the schizophrenia population has one of the highest rates of nicotine dependence. Cannabis is also among the most used recreational drugs by both the psychiatric and general population. Moreover, cannabis use in the psychiatric population has been shown to be double than in the general population (Bernett et al., 2007). Very often, people in general report the use of cannabis to overcome negative feeling or to "feel happier" supporting the "self medication" hypothesis of schizophrenia (Howes et al., 2004). In his paper, the authors formulate several hypotheses to explain these findings: 1. Psychotic patients are more likely to consume cannabis for the self medication hypothesis; 2. Cannabis could cause psychosis; 3. Cannabis could contribute to the risk of psychosis by precipitating a chain of events; 4. Cannabis could prolong psychotic illness in people already affected.

In order to further explore the role of environmental factors in the development of psychosis I aim to investigate the presence of an interaction between cannabis sativa and several markers within the Cannabinoid 1 receptor gene and the COMT gene in the aetiology of schizophrenia. The role of cannabis is also investigated to check for a main effect on this illness in a case-control analysis and for a mediation role. Literature on cannabis consumption and psychosis is reviewed in light of the main dopaminergic and endocannabinoid hypothesis in chapter 3 of this thesis.

CHAPTER 3

INTRODUCTION

CANNABIS SATIVA, THE ENDOCANNABINOID AND THE DOPAMINERGIC SYSTEMS

3.1 Cannabis Sativa and its origins

Cannabis is the world's most popular recreational drug. In Europe, use increased significantly across the continent in the last three decades of the 20th century, and it is now estimated that 40% of young Europeans have tried cannabis at least once in their life (United Nations Office on Drugs and Crime, 2006). Cannabis was first grown in Central Asia and then spread to the rest of the world. Nowadays, numerous names are given to cannabis, depending on the cultivation method, consumption method, plant strain and parts of the plant but traditionally, there were three main varieties: cannabis sativa (found in warmer climates like Thailand, Mexico and South Africa); cannabis indica (found mainly in Northern India) and cannabis ruderalis (less common and grown wild in Central Asia). In recent years, a fourth variety of cannabis has become popular; "sinsemilla" which refers to plants cultivated with a method which uses the non-fertilized female plant. In this way, the female plant grows to maturity without any contact with the male plant, producing flowers without seed; hence its name from the Spanish language "sinsemilla" "without seeds".

The various types of cannabis differ in their concentration of Δ^9Tetrahydrocannabinol (THC), the main psychoactive component, and the other major ingredient, cannabidiol (CBD); the latter does not impair cognition and may have anti-psychotic effects. During recent decades, certain types of cannabis have been re-engineered in order to maximize the level of THC, minimize the size of the plants and increase their strength for better survival. It is estimated that since the 1960s, the potency of street cannabis has almost doubled, partly due to plant crossing (United Nations Office on Drugs and Crime 2006). However, levels of THC may also vary depending on type of production and preservation techniques.

3.2 Cannabis and Psychosis

It is well known that in those with established psychosis, continued use of cannabis is associated with poor outcome and with more frequent and earlier relapses (Grech et al., 2005). More worryingly, a number of studies have indicated that exposure to cannabis is associated with cognitive impairment and increased risk of developing psychosis (Ashton, 2002; Arseneault *et al.*, 2004; Moore et al, 2007). As far back as 1987, Andreasson described an association between cannabis use and onset of schizophrenia (Andreasson *et al.*, 1987). This landmark cohort-study of more than 45,000 Swedish male conscripts (representing 97 percent of men age 18-20 in the population) and a 15-year follow up, found that heavy use of marijuana at age 18 increased the risk of schizophrenia later in life by six times. There was a dose-response relationship between cannabis use at age eighteen and the diagnosis of schizophrenia 15 years later. However only 3% of heavy cannabis users went on to develop schizophrenia, suggesting that cannabis might exert its causal role only in already vulnerable individuals. Since then a number of studies have investigated the association between cannabis and psychosis. These were summarised in a meta-analysis by Henquet et al. 2005a, which concluded that overall cannabis consumption was associated with a doubling of the risk of schizophrenia. More recently, Kuepper et al., conducted a 10 years follow up study, demonstrating that continued cannabis use, increased the risk of incident and persistent psychotic symptoms (Kuepper et al., 2011).

Verdoux et al. studied the interaction of cannabis use and psychosis in a non clinical population. The authors concluded that subject's level of vulnerability can modify the effect of cannabis such as subjects with high vulnerability are more likely to report adverse or unusual experiences during cannabis consumption (Verdoux et al., 2003). However, there was no evidence that cannabis use was increased following onset of psychotic symptoms (Verdoux et al., 2003).

Henquet et al., 2005b showed that baseline psychosis liability (attenuated psychotic symptoms and/or positive family history of psychosis) did not predict later cannabis use. Similarly, the longitudinal cohort study from Christchurch, New Zealand, which used statistical modelling in an attempt to distinguish between the causal and self-medication hypotheses, reported that the data were more compatible with a causal rather than a self-medication explanation. Thus, cannabis use increased risk of later psychosis, but the development of psychotic symptoms tended to decrease the subsequent consumption of cannabis (Fergusson et al, 2003).

Lately, Griffith-Lendering et al., proposed a model of bidirectional causal association between cannabis and psychosis. It was found that psychosis vulnerability at age 13, predicted cannabis use at 16 and 19 years of age (Griffith-Lendering et al., 2012). Furthermore, it was also found that cannabis use at 16 years of age, predicted psychosis vulnerability at 19 years of age (Griffith-Lendering et al., 2012). Most recently, Freeman et al., analysed 1714 individuals from the general

population, with the assumption that psychotic experience can occur as a quantitative trait in the general population (Freeman et al., 2013). Results showed a significantly higher level of persecutory ideation within the group of people using cannabis; furthermore, these subjects were twice as likely to report any paranoid ideation within the last month (Freeman et al., 2013).

3.3 Are some individuals at greater risk?

The above evidence suggests that cannabis consumption may have long term effects on mental health. Recent research has found that the risk is greater in those subjects who initially show some psychosis predisposition such as attenuated psychotic symptoms and/or positive family history for psychosis (Henquet al al., 2005b). Moreover, it appears that the timing of use may be important; use in adolescence seems to be more hazardous (Arsenault et al, 2002). These finding have also been confirmed by Estrada et al., who reported that subjects that were exposed to cannabis earlier, had an earlier onset of psychiatric disorders (Estrada et al., 2011). Dragt et al., also demonstrated that the younger the age of consumption, the earlier the onset of psychotic symptoms (Dragt et al., 2010). Genetic vulnerability is supported by evidence of a gene by environment (G x E) interaction between a functional polymorphism in the Catechol-O-Methyltransferase gene (COMT) and exposure to cannabis; the enzyme produced by the COMT gene has an important role in the breakdown of dopamine in the prefrontal cortex. COMT appears to moderate the influence of adolescent cannabis use on the development of adult psychosis, with a five-fold increased risk of developing schizophreniform disorder in cannabis users with the high activity Val allele (COMT) (Caspi *et al.*, 2005).

Furthermore, Henquet et al. in a double blind, placebo controlled cross over study with psychotic patients, relatives and healthy controls showed that carriers of the Val allele were more sensitive to psychotic experience induced by the compound and to worst results in memory and attention tasks; the same subjects showed prior psychometric psychosis liability (Henquet et al., 2006). Lately, Estrada et al., in the previous mentioned study, also reported that the Val allele carrier, showed an earlier onset of psychiatric disorders compared to Met carriers, therefore suggesting that the COMT rs4680 polymorphism modulates the interaction between cannabis and psychiatric disorders (Estrada et al., 2011). Such findings are supported by O'Tualthaigh et al., who conducted an animal study on COMT KO versus WT mice and found a gene X environment interaction (COMT X cannabis) on schizophrenia endophenotypes (O'Tualthaigh et al. 2012). The authors reported that COMT KO mice showed more vulnerability to the effects of cannabis on prepulse inhibition (PPI). Furthermore, cannabis adolescent exposure also had differential effects on anxiety and social behaviour in COMT KO mice versus WT mice (O'Tualthaigh et al. 2012). Acute inhibition of COMT also modified cannabis effects on startle and PPI reactivity (O'Tualthaigh et al., 2012).

Many studies, however, also failed to demonstrate the existence of an interaction between the COMT gene and schizophrenia. Munafo et al., found no association of rs4680 within the COMT gene and schizophrenia in a meta-analysis of case control studies (Munafo et al., 2005); furthermore Costas et al, also found no evidence of interaction between COMT haplotypes and cannabis in a case control study of schizophrenia (Costas et al., 2011). Zammit et al, in a well presented longitudinal study of 2630 individuals, on the interaction of COMT and cannabis in schizophrenia, found that cannabis was associated with self-reported psychotic experiences, but polymorphisms within the COMT gene, did not show any significant statistical interaction (Zammit et al., 2011). The COMT Val/Met allele also failed to be associated with Cognitive Remediation Therapy (CRT) in schizophrenia, thus not acting as a useful biomarker for cognitive improvement (Greenwood et al., 2011).

3.4 Experimental studies of the effects of cannabis on psychopathology

Acute cannabis intoxication can elicit transient psychotic symptoms. This has been documented in both clinical and experimental settings (D'Souza et al, 2004). Makela et al conducted a double blind, placebo controlled, and within-subject study in 19 healthy subjects. 5mg of sublingual Δ9-THC and placebo were administered and all subjects tested for spatial working memory using tasks known to be depended upon frontotemporal neural circuits (Makela et al., 2006). Results showed a significant increase in intrusion errors during spatial working memory tasks performance in males, however, in females, Δ9-THC seemed to enhance performance (Makela et al., 2006).

Leweke et al., studied the effect of nabilone, a psychoactive synthetic 9-trans-ketocannabinoid and cannabidiol in a group of healthy volunteers on binocular depth inversion and behavioural state (Leweke et al., 1999). It was found that an impairment of binocular depth inversion after nabilone administration was attenuated by subsequent administration of cannabidiol (Leweke et al., 1999).

Henquet et al., conducted a double blind, placebo controlled cross over study with psychotic patients, relatives and healthy controls. All subjects were exposed to 300μg/Kg of Δ9-THC in tobacco cigarettes or placebo and assessed with cognitive tests done by computer (Henquet et al., 2006). Tests were used to assess verbal and visual memory and attention and were analysed together with COMT genotyping data (Henquet et al., 2006). Results showed that Δ9-THC generally worsened performances on cognitive tests and that, carriers of the Val COMT allele were more sensitive to psychotic experience induced by the compound and to worst results in memory and attention tasks if they showed prior psychometric psychosis liability (Henquet et al., 2006). Data thus suggest that the COMT gene and particularly the rs4680 SNP may moderate the effect of Δ9-THC, where pre-existing psychometric psychosis liability exists (Henquet et al., 2006).

Pisano et al., in 2006 demonstrated that in hippocampus and prefrontal cortex an increase in dopaminergic activity following administration of THC is observed (Pisanu et al., 2006).

Morrison et al., completed a randomized double blind study of the effects of intravenous THC (2.5mg) in 22 psychiatrically well male subjects (Morrison et al., 2009). Similar to the findings of D'Souza and colleagues, THC elicited a transient increase in positive psychotic symptoms as measured by the PANSS (Morrison et al., 2009). Participants also rated themselves on the CAPE scale (Community Assessment of Psychic Experiences; Stefanis et al 2002) and a reasonable correlation in psychosis scores between both scales (Spearman's rho, $r= 0.63$, $p<0.001$) was observed (Morrison et al., 2009). The same author also found that THC elicited schizophrenia-like negative symptoms in a study of 22 psychiatric well male subjects, after intravenous administration of THC (2.5mg) (Morrison and Stone, 2011). Plasma levels of THC could be compared to the ones observed in recreational use (Morrison and Stone, 2011). Englund et al., in a between-subjects design study, found that in subjects who were administered CBD before THC, psychotic symptoms were less likely to occur compared to those who had a placebo before the THC; post-THC paranoia was also less present in the CBD group (Englund et al., 2013). Furthermore, the same group of subjects experienced less memory impairment (Englund et al., 2013). CBD appears therefore to moderate the effect of THC-elicited paranoid symptoms and memory impairment.

3.5 Endocannabinoid Mechanisms

The psychotogenic properties of cannabis are, almost certainly, attributable to Δ-9-tetrahydrocannibinol (THC) via stimulation of the CB1 receptors in the brain.

The CB1 receptor is the most common G-protein coupled CNS receptor. Levels are high in the frontal cortex, the basal ganglia, the hippocampus and the cerebellum. (Perhaps not coincidentally, each of the aforementioned regions has been proposed as a locus for the pathophysiology of schizophrenia, at one time or another).

Studies of the hippocampus and cerebellum have been invaluable in establishing general features of the endogenous cannabinoid (endocannabinoid, eCB) system (Kawamura et al., 2006; Matyas et al., 2006; Uchigashima et al. 2007; Eggan et al. 2000). Briefly, principal output neurons (Purkinje cells, pyramidal neurones) synthesize and release endocannabinoids (eCBs) from their dendritic spines. Released eCBs stimulate CB1 receptors on neighbouring pre-synaptic terminals, which couple to $G_{i/o}$ proteins and inhibit neurotransmitter (NT) release. Whilst short-term inhibition of NT release appears to be mediated by a direct effect of βγ subunits on Ca^{2+} and K^{+} currents, sustained inhibition requires effects on pre-synaptic intracellular signalling pathways (cAMP, MAPK). Across all the major brain structures where eCB signalling has been explored, both glutamatergic (excitatory) and GABA-ergic (inhibitory) terminals are direct targets for endocannabinoids. There is considerable current interest in exploring how eCBs are synthesized under physiological conditions and how different patterns of synthesis evoke short or long-term pre-synaptic inhibition.

Endocannabinoid dependent long-term depression (LTD) of synapses has been demonstrated in the hippocampus, the amygdala, the cerebellum and the ventral and dorsal striatum. At the behavioural level, it has been demonstrated that eCB signalling is necessary for new learning in the cerebellum and amygdala. In sensory cortices, eCBs are essential mediators of spike-timing dependent LTD (Bailey et al., 2000; Freund et al., 2003).

Recently it has become apparent that the endocannabinoids function as crucial molecular cues in the development of the foetal nervous system (Harkany et al. 2007; Harkany et al. 2008). Endocannabinoids stimulate the proliferation of neural progenitors, provide instructions for glial cell fate, direct the migration of immature neurones and regulate axonal growth, pathfinding and target selection (Aguado et al. 2006; Berghuis et al. 2005; Berghuis et al. 2007; Mulder et al. 2008). In immature networks, where GABA is excitatory, eCBs function as key inhibitory transmitters (Bernard et al. 2005). Animal work has shown that perturbation of the endocannabinoid system during early development, either pharmacologically with agonists or antagonists or genetically, can lead to marked alterations at the cellular and circuit levels, (Bernard et al. 2005; Harkany et al. 2007), and in-uterine exposure to CB1 receptor agonists can elicit long-term changes which impact on learning and memory performance in later life (Antonelli et al. 2005; Mereu et al. 2003). Given that the eCBs are involved in fundamental neurodevelopmental processes, it seems intuitive that human brains exposed to cannabis in-utero might show catastrophic, long-lasting psychopathological impairments. The available evidence does not support this; instead the long-term effects of prenatal cannabis appear to be subtle, suggesting that compensatory molecular pathways may have developed (Fried and Smith 2001; Huizink and Mulder 2006). Several animal studies have investigated the effects of prenatal cannabis exposure (Antonelli et al., 2005) (Castelli et al., 2007) (Ferrari et al., 2009), thus reaching the conclusion that systematic cannabis exposure during the prenatal period, indeed leads to changes observed in behaviour and brain of the offspring. Two major studies, the Ottawa Prenatal Prospective Study (OPPS) and The Maternal Health Practices and Child Development Study (MHPCD) have followed up cohorts of children born to mothers who smoked cannabis (heavily) during pregnancy. Findings from the OPPS showed that in-uterine exposure to cannabis had no effect on the full-scale IQ as measured by the WISC-III (Fried 2002). However children exposed to cannabis prenatally had an excess of impulsive, hyperactive and delinquent behaviours (Fried et al. 1992; Goldschmidt et al. 2000; Leech et al. 1999), displayed poorer performance in tasks of verbal memory and executive functioning (Fried 2002; Huizink and Mulder 2006) and showed poorer attainment of reading skills (Goldschmidt et al. 2004). The effects of prenatal exposure to cannabis may operate by impacting on prefrontal performance in later life (Fried 2002).

More recently, DiNieri et al., examined striatal dopamine and opioid genes in human fetal subjects exposed to cannabis (DiNieri et al., 2011). The author showed that D2 (DRD2) receptor mRNA expression was decreased in human ventral striatum (DiNieri et al., 2011). These findings were

taken further by modelling a study with mice. Pregnant rats were exposed to THC and the offspring examined. Results showed decreased DRD2 expression and reduced D2R binding sites in offsprings (DiNieri et al., 2011).

There has been less work on whether prenatal cannabis exposure predisposes to later psychiatric morbidity. Findings from the MHPCD show that in-utero cannabis exposure predicts depressive symptomatology at 10 years of age (Gray et al. 2005) and pre-natal cannabis exposure may predispose to subsequent illicit drug misuse (Day et al. 2006; Spano et al. 2007). El Marroun et al., studied the relationship between prenatal cannabis exposure and aggressive and attention behaviour in offspring. (El Marroun et al., 2011). Results showed that there was indeed a relationship between prenatal cannabis exposure and behavioural problems in children, however only in girls in the aggressive behaviour and attention problems areas (El Marroun et al., 2011). Psychiatric morbidity could also be entirely related to home environment and would need further research. As yet, no studies have examined whether there is an association between prenatal cannabis exposure and the subsequent emergence of psychotic disorders. This is clearly an important question for further study. Furthermore, alternative genetic mechanisms may contribute, including maternal genetic effects or environmental factors that interact with genetics to cause schizophrenia. Maternal genetic risk factors could also mediate cannabis exposure, which in turn could influence in utero environment early during neurodevelopment.

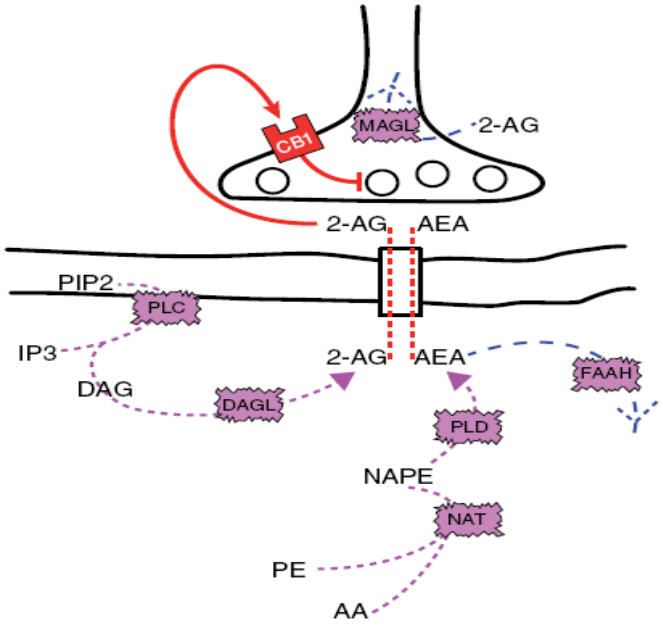


Figure 3.1 shows the retrograde transmission. Endocannabinoids are synthesized and released post-synaptically inhibiting neurotransmitter release from pre-synaptic terminals (Morrison and Murray, 2007)

3.6 Endocannabinoids and Psychosis

The above evidence points towards the endocannabinoid system being impaired and playing a role in schizophrenia. It has been suggested that eCB impairment may be a proximal pathology in some forms of schizophrenia. As yet, post-mortem and PET studies of altered CB1 receptor availability in schizophrenia have been equivocal. Studies of the genetic variation in components of the ECS have begun to address this speculation, however, the CB1 receptor remains the most extensively examined. An AAT-repeat microsatellite in the 3' flanking region of the CNR1 gene has been reported to be associated with the hebephrenic sub type of schizophrenia in a Japanese population (Ujike et al., 2002), a finding partially replicated in a recent study (Chavarría-Siles et al, 2008). However, another study failed to find an association between schizophrenia and this microsatellite or with other polymorphisms within the gene (Seifert et al., 2007). This study (Seifert et al., 2007) also failed to identify any non synonymous polymorphisms after sequencing the coding regions of the CNR1 gene of 50 schizophrenic patients. Negative results also came from the study of Zammit et al., where no evidence of genotype effect was found in CNR1 or COMT in a case-control study of schizophrenia (Zammit et al., 2007). There are, however, evidences of an interaction between polymorphisms within the CNR1 gene and cannabis use, as shown by Ho et al., in a study of 235 schizophrenia patients (Ho et al., 2011). The authors investigated changes in white matter and cognitive deficits in schizophrenic patients exposed to cannabis. Results were suggestive of an interaction between a SNP within the CNR1 and cannabis use, in that, patients with the risk allele and positive cannabis use, showed smaller fronto-temporal WM volumes and worse performances in neurocognitive tasks (Ho et al., 2011). In the same cohort, there was also found a significant main effect of MAPK14 CNR1 diplotype and a G x E interaction with cannabis on WM brain volumes in patients exposed to cannabis. This effect seemed to be additive (Onwuameze et al., 2013). As suggested by the authors, MAPK phosphorylation increase precedes CNR1 induced apoptosis, there could therefore be, a genexgene interaction as the basis of the structural abnormalities in white matter observed in this group of patients exposed to cannabis (Onwuameze et al., 2013). The CNR1 gene has also been associated with weight gain in schizophrenia by several studies throughout the years. Recently Yu et al., showed an association of several polymorphisms within the CNR1 gene and metabolic syndrome in schizophrenia (Yu et al., 2013). Tiwari et al., also showed association between a SNP on the CNR1 gene and weight gain in patients treated with clozapine and olanzapine (Tiwari et al., 2010). The latest GWAS however, found no association between schizophrenia and the endocannabinoid system. This is suggestive of the presence of possible false positive in the above mentioned studies. On the other hand, other measures of the ECS, such as endogenous ligands have shown promising results, giving rise to the biological plausible endocannabinoid hypothesis.

Altered levels of anandamide, the endogenous compound of the ECS, have also been linked to schizophrenia. In a study of first-episode antipsychotic-naïve schizophrenic patients, Giuffrida et al (2004) found that CSF levels of anandamide exceeded by 8 times those of healthy controls. More recently, Leweke et al (2007) showed that CSF anandamide levels were altered in patients with low frequency and high frequency cannabis use. This was not observed in the control group, suggesting that use of cannabis and indeed frequency of exposure could contribute to the alteration of anandamide signaling in susceptible individuals (Leweke et al., 2007). An important key point in the mechanism of interaction between cannabis consumption and psychotic symptoms could therefore be subsequent changes in levels of AEA. Parolaro et al., suggested a possible explanation in the down-regulation of AEA caused by excessive consumption of cannabis (Parolaro et al., 2010). Endocannabinoid levels disregulation have also been reported in mice models (Vigano et al., 2009) (Seillier et al., 2009).

While the main component of cannabis, Δ^9Tetrahydrocannabinol (THC), is believed to be responsible for the psychotic like effects that follows acute cannabis intoxication, cannabidiol (CBD) is thought to exert antipsychotic effects. In preclinical studies, CBD showed similar antipsychotic activity to haloperidol but with a lack of toxicity (Zuardi et al., 1991). Partial improvement of symptomathology of schizophrenic patients has also been observed in small clinical trials (Zuardi et al., 1995) (Zuardi et al., 2006). Moreover, when co-administered, CBD can reverse the psychotropic effect of THC (Zuardi et al., 1982). More recently, as mentioned earlier, Englund et al., showed that Cannabidiol (CBD) can inhibit THC-elicited paranoid symptoms and memory impairment (Englund et al., 2013). To date, the neurobiology of this mechanism remains still unclear, but CBD is being studied as a possible and less toxic alternative to antipsychotics. To date there have been many studies on animal models of CBD on mice and rats (Long et al., 2010) (Moreira and Guimaraes, 2005) (Malone et al., 2009) to cite a few. The present view, as expoused by Zuardi et al., is that CBD behaves as an atypical antipsychotic in preclinical studies, improving symptoms but not impairing motor functions of subjects (Zuardi et al., 2012). Its action could depend on the facilitation of glutamate activity in the prefrontal cortex, via increase in anandamide-mediated CNR1 activation, thus causing glutamate activity to decrease (Zuardi et al., 2012).

3.7 Interaction between Endocannabinoids and Dopamine

The dopamine hypothesis of schizophrenia, dating back to the 60s, suggests that up-regulation of dopamine transmission in the brain is the underlying cause of the disorder. In 1996, Laurelle et al., showed that psychotic patients released excessive striatal dopamine after an amphetamine challenge; this positively correlated with the severity of symptoms (Laurelle et al., 1996). In 2005, Kapur et al. proposed the 'motivational salience theory' which postulates that striatal dopamine

release directs the process by which stimuli are given significance. Abnormal dopamine release leads to excessive significance being attributed to stimuli, hence the formation of delusions to explain such seemingly important yet confusing experiences (Kapur et al., 2005). Mice models have also been used to try to establish the effect of various known risk factors for psychosis on dopamine function. Seeman et al. (2005) showed that high affinity to the D2 receptors could be explained by a range of different factors ranging from Gene knock-outs, to obstetric events and to drug use. Up-regulation of dopamine seems more likely to be the final 'common pathway' rather than the underlying cause of schizophrenia.

Interactions between the DA and eCB systems are extensive (and complex) but several key (and unusual) findings are outlined here which at least illustrate the close relationship between DA and eCB signalling.

It is well established that DA acting at D2 receptors in the striatum is necessary for eCB-dependent LTD of corticostriatal fibres, although at present the functional significance of corticostriatal LTD remains theoretical. At the behavioural level, repeat administration of THC evokes cross-sensitisation to the effects of amphetamines. Furthermore, recent work indicates that CB1Rs have a role in the development of amphetamine sensitisation, (although diametrically opposed findings with receptor knockout compared to pharmacological blockade, hinders any explanation at this stage). Utilising in-vivo voltammetry, Cheer and colleagues (2004) have shown that the CB1 inverse agonist SR141716A, (rimonabant) inhibited cocaine evoked DA release in the striatum.

It appears that the two neurochemical systems interact most intimately at the level of D2-CB1 dimerisation: Mackie and colleagues (1995) have shown that concurrent stimulation of both receptors leads to formation of a D2-CB1 protein complex. Remarkably, (and in stark contrast to the 'traditional' intracellular effects of D2 and CB1) the hetero-dimer preferentially coupled to Gs, and increased cAMP levels. Although the implications of this finding have yet to be appreciated, one possibility is that, in the presence of THC, striatal D2 receptors behave (in biochemical terms at least) like D1 receptors.

Thus, it seems that endocannabinoids modulate (excitatory and inhibitory) inputs to dopaminergic neurones. This is of great interest since the epidemiological evidence suggesting an interaction between cannabis and COMT genotype in conferring risk for schizophrenia, implies that dopamine is involved (at some level) in the psychotogenic effects of THC. In an elegant experimental study, Henquet and colleagues (2006) showed that COMT genotype determined (in-part) whether healthy volunteers experienced an acute psychotic reaction following a standardised cannabis 'joint'. Psychotic outcomes were associated with the *Val* allele providing experimental support for the epidemiological findings of Caspi et al (2005).

It has become clear that in animal studies CB1 agonists elicit burst-firing of midbrain DA neurones and increase striatal dopamine levels. The COMT *Val* allele appears to be associated with increased DA synthesis in VTA neurones. One suggestion is that individuals possessing the *Val/Val* genotype are more likely to experience THC-psychosis, because their DA systems are already 'primed'. However, further work is needed to confirm both the epidemiological and experimental studies implicating a cannabis-COMT interaction.

CHAPTER 4

MATERIALS AND METHODS

This chapter contains a description of the main hypothesis of this work and illustrates the recruitment strategy for cases and controls as well as study sample characteristics... Hypotheses are explained in more detail in each experimental chapter (Chapters 5-8). This chapter also contains detailed methodology on DNA handling and general methodology on laboratory techniques used to analyse samples. Details on laboratory procedure for each technique are given in chapter 6 (TaqMan Genotyping Assay) and 8 (Microsatellite Analysis).

Statistical methodology is reported in detail in each of the experimental chapters. This includes a summary of analytic approaches used, statistical software used, command lines used and description of output files.

4.1 Main Hypothesis

This is a case-control study in a cohort of hospital based psychotic patients and healthy matched controls.

The goal of this study is to investigate the role of the CNR1 and the COMT gene in psychosis. In light of the published literature and knowledge of the biology of the Endocannabinoid and Dopaminergic Systems (outlined in the introductory chapters), I hypothesize that common variations within the CNR1 and the COMT gene play a role on the pathophysiology of psychosis and may also give an increase risk for the disorder by interacting with cannabis use.

The two main areas of investigation of this work are:

The involvement of common variants within the CNR1 and the COMT gene in the aetiology of psychosis

The interplay of the CNR1 and the COMT gene with the environment - cannabis use- to give an increase of risk for psychosis.

4.2 Samples under investigation

The samples under investigation are part of two large studies on first episode of psychosis: the Psychosis Incident Cohort Outcome Study (PICOS) and the Genetics and Psychosis study (GAP). The GAP Study is based in London at the Institute of Psychiatry, department of Psychosis Studies. The PICOS Study is based in Verona, Italy at the department of Psychiatry at Verona University. Both study samples consists of first episode psychosis subjects and non-psychotic volunteers. The recruitment strategy for both studies is a hospital-based incident case study for first episode patients. In the next sections there are details on cases and controls recruitment.

4.2.1 The Genetics and Psychosis Study (GAP)

4.2.1.1 Patients Recruitment

Subjects were recruited from five hospital/health centres: Maudsley Hospital (Eileen Skellern 1, Eileen Skellern 2, Jim Birley Unit, Douglas Bennett 2 and Douglas Bennett 3); Guy's hospital (Ruskin ward, John Dixon ward); Bethlem Royal Hospital (Alexandra ground floor ward, Gresham Psychiatric Intensive care unit, Gresham 1 & 2 wards); Lambeth Hospital (Lambeth Early Onset inpatient unit); and Croydon (COAST – Early Intervention Psychosis Service). The strategy for recruitment consists of regular contact of inpatients services by researchers of the GAP group. When a case of first episode of psychosis is identified, the subject is approached on site and carefully explained about study aims and assessment involved. The subject is then included in the study if all inclusion criteria are fulfilled and he or she agrees. Inclusion criteria are: 1. English fluency; 2. South East London residency; 3. Age between 18 and 65 years; 4. Being at the first episode of psychosis; 5.Subjects are within three months of the first contact with the psychiatric services. The exclusion criteria are: 1. Poor English fluency; 2. Psychosis due to acute intoxication; 3. Suspected organic psychosis; 4. Presence of any disability that could impair judgment on giving consent for the study; 5. Age below 18 years. Subjects needed to consent to be sampled for DNA (through phlebotomy or collection of buccal cells) as this is the minimum requirement for participation in the study. Assessments were conducted at the various hospitals or at the IoP throughout several sessions. All subjects were reimbursed for their time according to ethical permission n. 0546734 and had the right to end their collaboration with the study at any time.

Diagnosis was confirmed by the clinical research team through consensus meeting carried out by groups of four psychiatrists. Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (World Health Organization Assessment, Classification and Epidemiology, Geneva 1999), Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) and notes obtained from the electronic Patient Journey System are taken into consideration in order to reach a consensus diagnosis. SCANs and PANSS assessments are carried out by trained researchers of the GAP group.

4.2.1.2 Controls Recruitment

Healthy volunteers were recruited in the south east London area (i.e. the same area as the patients). The study was advertised in local newspapers, job centres and two volunteer databases: Mindsearch (www.mindsearch.com) and Biotrax (www.biotrax.com). Prior to inclusion in the study, all volunteers were screened for psychosis by researchers of the GAP group by using the Psychosis Screening Questionnaire (PSQ) (Bebbington et al., 1995). Subjects were excluded if they scored positively for psychosis or for history of mental disorders or if sustained head injury or if undergoing hormonal treatment.

If the Psychosis Screening Questionnaire did not outline any reason for exclusion from the study, subjects were asked contact details and joined a queue. Researchers would then call back and arrange a suitable time for meeting. All healthy individuals needed to also consent to be sampled for DNA (through phlebotomy of collection of buccal cells) as this was the minimum requirement for participation in the study. Assessments were carried out by researchers of the GAP group at the IoP in several sessions and subjects had the right to withdraw consent at any time.

All subject recruited were carefully matched with the disease population for ethnicity, socio economical status, gender and age.

4.2.1.3 Socio-demographic questionnaire

The socio demographic questionnaire used in the present study is a modified version of the MRC Sociodemographic Schedule (Mallett et al., 2003). The SDS questionnaire was used for both cases and controls in order to collect data regarding age, gender, self rated ethnicity, level of education achieved and employment status. It comprises 19 items with multiple choice answers rated by the researchers. The Family Interview for Genetics Study (FIGS; Maxwell, 1992) questionnaire was also used. The FIGS allows collecting specific data regarding the family pedigree of probands and controls by asking the birth place of parents and grandparents. It also helps tracking migration status.

In this study, all Caucasian subjects were grouped together including those reported to be British or white other by the questionnaire and all Black African, black Caribbean and mixed ethnicity with either black African, black Caribbean or black British were also grouped together. Subjects were therefore divided in two main groups: Caucasian and Black/mixed ethnicity when the study sample was ethnically stratified. When data analysis was carried out in the whole GAP study sample, all other ethnicities were included (Further details are given in the “Sample characteristics” section).

4.2.1.4 The Cannabis Experience Questionnaire (CEQ)

Cannabis data were obtained by a screening questionnaire used for both cases and controls. The questionnaire is the revised version of the Cannabis Experience Questionnaire (Barkus et al., 2006) and it includes date of first use, preferred mode of use, frequency as well as data on self reported feelings before and after consumption. The Cannabis experience questionnaire contains 19 items with multiple choice answers as well as an open answer. From item 14 onwards it collects perceived experiences while smoking and after. It also establishes type of cannabis used and quantity smoked during adolescence and adulthood. The questionnaire also collects information on other drugs used in combination or during the same period of time as cannabis consumption, this helps to correct data for possible confounders. The GAP Cannabis Experience Questionnaire is further explained in chapter 7 with reference to literature available and hypothesis under investigation.

4.2.1.5 Sample characteristics

The Genetics and Psychosis Study sample consisted of 516 participants: 169 non psychotic controls and 347 first episode patients. Cannabis data (cannabis ever used yes/no) were available for 459 individuals: 312 cases and 147 controls.

In this work and for all statistical analyses the GAP sample has been divided into two groups:

Caucasian group

Black group

The two groups have always been analysed separately unless otherwise stated.

The Caucasian group consisted of 219 individuals and the Black group (black Caribbean, black African and mixed ethnicity with either black Caribbean or black African) consisted of 208 individuals. All individuals (89) with any other self reported ethnicity were excluded from any analysis. A summary of the GAP Study samples characteristics is shown in table 4.1.

Further details on the number of individuals included in each analysis are given in each experimental chapter.

Table 4.1 GAP Study sample demographics

	total number	male %	female %	Cannabis users %
Caucasian cases	**174**	**65.7**	**34.3**	**39.1**
Caucasian controls	**45**	**55.8**	**44.2**	**44.4**
Black cases	**113**	**69.9**	**30.1**	**34.5**
Black controls	**95**	**68.8**	**31.2**	**30.5**

4.2.2 The Psychosis Incident Cohort Outcome Study (PICOS)

4.2.2.1 Patients Recruitment

First Episode Psychotic patients were recruited by researchers of the PICOS study in Veneto Region, in North-East Italy. A representative cohort of subjects presenting at services with psychotic symptoms were included in the study from January 2005 to December 2008. Follow up was also carried out at 1 year, 2 years and 5 years. Inclusion criteria were: 1. Age between 15 and 54 years old; 2. Being resident in the Veneto Region Community Mental Health Services catchment area; 3. presence of (a) *at least 1* of the following: hallucinations, delusions, qualitative speech disorder, qualitative psychomotor disorder, bizarre or grossly inappropriate behaviour, or (b) *at least 2* of the following: loss of interest, initiative and drive, social withdrawal, episodic severe excitement, purposeless destructiveness, overwhelming fear, marked self-neglect; 4. Being at the first contact with psychiatric services and not having received treatment for psychosis for more than 3 months. Patients were excluded from the study if they met the criteria for organic brain disease.

Patients were assessed with a set of standardised measures as follow:

Schedule for Clinical Assessment in Neuropsychiatry (**SCAN**; WHO, 1992)
Psychosocial and Environmental Stressors (APA, 1994), the Axis IV of DSM-IV,
Scale for the Assessment of Positive and Negative Symptoms **(PANSS**, Andreasen, 1984)
Family Interview For Genetic Studies **(FIGS**; Maxwell, 1982)

4.2.2.2 Controls Recruitment

Non psychotic controls were recruited via the Blood Transfusion Service from the same catchment area of Verona, Italy. Subjects were excluded if personal or family history of psychotic disorders was positive, they were assessed with the SCID-NP (Spitzer et al, 1992) and the Family Interview for Genetics Study (FIGS; Maxwell, 1992). Data on self reported ethnicity was also collected to match cases and controls. Controls were recruited from the same area as cases; they were not matched for socio economic status.

4.2.2.3 Sample characteristics

The PICOS Study samples consisted of 654 participants (347 cases and 307 controls) of Italian Caucasian origins (99%). Cannabis data (Lifetime cannabis use yes/no) were only available for psychotic patients as non psychotic controls were recruited via the Blood Transfusion Centre (BDC) in Verona (Italy) and always tested negative for cannabis use during routine tests run by the BDC. No cannabis data were therefore used in any of the statistical analyses. No gender information was available. Details about numbers of subjects available for each statistical test are reported in each of the experimental chapters.

4.3 DNA handling

4.3.1 DNA extraction from blood

Blood samples were collected using 1x 6ml EDTA tubes. DNA was extracted using the standard phenol chloroform extraction protocol. During the first day the EDTA tubes containing blood were placed in the cold room and subsequently the blood poured into a 50ml Falcon tube and made up to 45ml by adding red cell lysis buffer (RCLB). They were then mixed vigorously and placed on a shaker for 20mins. Falcon tubes were centrifuged at 1600rpm 25mins 18^{o}C and carefully decanted to discard the supernatant. Tubes were then left upside down on tissue to get rid of the last of the red cell debris. The pellet was resuspended in 1ml lysis buffer Slagboom and 2X (2ml per 100ml) and transferred to 15ml Falcon tube. They were placed on a rotator overnight (180rpm).

The second day the Falcon tubes were incubated in a water bath at 65^{o}C for 2.5 hours. The contents were poured into a single15ml tube and 0.25ml Majik Mix were added then all tubes were caped and again shaken vigorously. Tubes were subsequently centrifuged at 13000rpm for 20mins at 18^{o}C.

The supernatant was decanted into fresh 15ml tubes and pellet discarded. 800uL of 100% isopropanol were then added; tubes inverted for 30 minutes and then again centrifuged at 13000rpm for a total time of 20mins at 18^{o}C of temperature. On rack they were tipped upside down and supernatant discarded. Tissue paper was used to pat the top of the tubes before leaving them upside

down for a couple of minutes, then 1ml 70% ethanol was added. Tubes were then centrifuged again at 13000rpm for 20mins at 18°C and the supernatant discarded. Tubes were then left to dry on tissue paper for approximately 1.5 hours. Finally 980μL of TE were added and tubes placed in incubator oven overnight.

Red Cell Lysis Buffer:

10mM Tris HCl pH ~7.6

5mM $MgCl_2$

10mM NaCl

Lysis Buffer

100ml	NaCl	1M	
10ml	Tris HCl	1M	pH 8
20ml	EDTA	0.5M	pH 8 (EDTA will not dissolve until pH 8)
50ml	SDS	10%	
820ml	dH_2O		

Add 2% Proteinase K just before use.

4.3.2 DNA extraction from cheek swabs

Cheek swabs kits are created as follow: 2.5ml of Lysis buffer with Proteinase K was inserted in a 15ml Falcon tube. Ten cheek swab buds are then placed in each tube. The dose for 100 kits is 250ml lysis buffer and 2.5ml Proteinase K.

Proteinase K is consists of 1g Proteinase K (from Sigma) $\Rightarrow$ 50ml dH_2O which is then aliquoted into 1ml and frozen.

4.3.3 DNA volume measurement and quantification

DNA volume for all extracted samples was calculated by weighing each sample on a digital scale in the laboratory. Firstly the tare was calculated by measuring the weight of an empty tube of the same kind, then one by one, all samples were weighed. Assuming that the 1ml of DNA at 4 °C

weighs 1gram, values expressed in grams on the scale were then simply changed to ml and then multiplied by 1000 in order to obtain values in μl.

All data were entered in an excel file and subsequently in SADMAN, a smart database system created by Bernard Freeman (unpublished in literature and only used at the SGDP facility, Institute of Psychiatry, KCL) that allows fast tracking of individual samples as well as entire plates. SADMAN also stores information regarding date of extraction, date of quantification, methodology used and up to date volume and concentration of each sample. Quality control was performed in three plates and no discrepancies were found.

DNA concentration was measured using the UV- Vis Spectophotometer NanoDrop machine from Thermo Scientific. The retention system used by the NanoDrop 2000 (UV-Vis Spectophotometer, Thermo Scientific) analysed very small quantities of DNA, the range can vary between 0.5μl to 2.0μl. In this study it was decided to use 1.5μl because there is a consistent less possibility that air bubbles get inserted in the machine causing errors and therefore waste of DNA due to forced repetition of the measurement. All samples were decapped and prepared for the reading. The arm of the machine was cleaned with ddH_2O and then vigorously wiped with laboratory paper wipes. Subsequently, 1.5μl of ddH_2O was inserted in the arm in order to initiate the process and 1,5μl of ddH_2O for a second time in order for the software to establish the blank measure. 1.5μl was then taken from each sample and pipetted onto the arm; the pedestal was then moved down to adjust for path length (0.05mm – 1mm) and then the arm was wiped clean. In order to obtain minimise errors; each sample is entered on the computer before measuring. This procedure was repeated for each sample. Quality control was carried out for 40 samples and discrepancies were not significant. Data are stored in the software of the NanoDrop and acquired in Tab format simply by using a USB memory and then converted into excel format in order to be analyzed. Data of all samples have been entered in SADMAN and are now available together with all information for each sample.

All samples were diluted in ddH_2O by using the Tecan Freedom Evo 200 Maschine (Tecan Integration Group) at the SGDP laboratory facility.

Work plates were created using matrix storage plates of 96 wells and samples were diluted to 10ηg/μl. Firstly a FX file was created in order to exactly calculate volumes and concentrations of each sample. To do this, information for SADMAN were used and then the file runs in the Tecan maschines computer. Few samples (around 10) were less concentrated due to problems during DNA extraction; they were kept at their original concentration and did not cause problems during genotyping for the majority of cases. Plates were stored at -20^{o}C or at 4 ^{o}C during the day when experiments were being carried out.

4.3.4 DNA storage

All samples are bar-coded, entered in SADMAN database system and stored at -80ºC in freezer at the SGDP centre. Barcodes are created in sequence by the manager of the SGDP laboratory at the IoP after request is made by the GAP group. Barcodes are made of letters and numbers and are matched to progressive numbers on original folders stored at the main building of the IoP in order to facilitate tracking. Patients and controls are randomly assigned barcodes so that laboratory work can be blind and not be subject to bias. Clinical assessments of patients and controls can only be matched by following identification numbers present on paper folders. DNA is stored in storage matrix 96 wells plates and each tube is individually barcoded as well as the plate with its own identification name. All barcodes are then scanned into SADMAN and subsequentely all plates were stored at -80ºC in freezers at the SGDP laboratory centre.

4.4 SNPs selection

4.4.1 SNPS within the CNR1 gene

Markers of the CNR 1 gene have been selected using the international HapMap data and haploview program for tagging SNPs (Barrett et al., 2005).

The HapMap is a catalog of common genetic variants that occur in humans.

The construction of the HapMap occurs in three steps

(1) Single nucleotide polymorphisms (SNPs) are recognized in DNA samples from various individuals.

(2) Adjoining SNPs that are inherited together are compiled into "haplotypes"

(3) "Tag" SNPs inside haplotypes are recognized that exclusively identify those haplotypes. By genotyping the three tag SNPs; researchers can recognize which of the four haplotypes are present in each individual.

HapMap data have been generated thanks to the Human Genome Project and is now a public database of common variation in the human genome, accounting for more the a million Single Nucleotide Polymorphisms. Data on SNPs have been obtained by genotyping 269 DNA samples of four populations: CEU (collected from the CEPH for Utah residents with European ancestry), JPT (Japanese population from Tokyo), CHB (Chinese population from Beijing) and the YRI (Yoruba from Nigeria). Data generated by the HapMap document the generality of recombination hotspots, the structure of linkage disequilibrium and low haplotype diversity (The International HapMap

Consortium, 2005). The advantage of using the HapMap data is that SNPs are presented graphically in a linkage disequilibrium plot, so that only a subset of them needs to be genotyped.

In Phase II of the HapMap consortium released in 2007, 2.1 million SNPs were added to the original map, in the same individuals. This certainly improves choice on tag SNPs giving also a better understanding of genetic variation (The HapMap Consortium www.Hapmap.org).

In the latest phase, the HapMap consortium also added seven more populations to the four already genotyped: Maasai in Kinyawa, Kenya; Luhya in Webuye, Kenya; Chinese in metropolitan Denver, CO, USA; Gujarati Indians in Houston, TX, USA; Toscani in Italia (Tuscans in Italy); African ancestry in the Southwest USA; and Mexican ancestry in Los Angeles, CA, USA. These populations were both genotyped for 1.6 million SNPs and sequenced in 2Mb of the ENCODE II regions (The HapMap Consortium www.Hapmap.org).

After choosing the gene and the reference population, the HapMap web page produces a diagram of the gene with all SNPs present in the exact area under investigation.

Finally, Tag SNPs have been selected using the Haploview software. Haploview is a bioinformatic software that allows the analysis of patterns of linkage disequilibrium (LD) in a given set of genetic data (Barrett et al., 2005). Details on setting used in this thesis are in chapter 5. Linkage Disequilibrium between SNPs within the CNR1 gene and the COMT gene was computed using Haploview version 3.32 (Barrett et al., 2005). Haploview only computes pairwise LD statistics for markers within a certain distance of each other, the accepted default value is set to 500K (Barrett et al., 2005). Haploview excludes individuals with less than 50% complete genotypes and generates LD blocks only if 95% of informative comparisons are "strong LD". Markers with MAF < 0.05 are therefore ignored by default (Barrett et al., 2005).

The AATn microsatellite has been chosen because of its previous association with schizophrenia described in the literature publicly available. Details are given in chapter 8.

4.4.2 SNPs within the COMT gene

Markers within the COMT gene were chosen because they were considered logical candidates. They have all been previously described in literature. Details are given in chapter 6 and 7.

4.5 Laboratory techniques

4.5.1 TaqMan Genotyping:

TaqMan® SNP Genotyping Assays from Applied Biosystems is based on 5' chemistry and amplifies specific SNPs in genomic DNA samples. Most of the assays were pre-designed and validated by Applied Biosystems apart for one SNP further discussed in chapter four. As shown in Figure 7, the TaqMan probe contains two types of dye: a reporter dye located at the 5' end and a quencher dye, located at the 3' end. The reporter dye is separated from the quencher dye during the reaction. The fluorescence increase can be detected only if the target sequence is present and has been amplified during the PCR reaction. Non specific amplification is not detected.

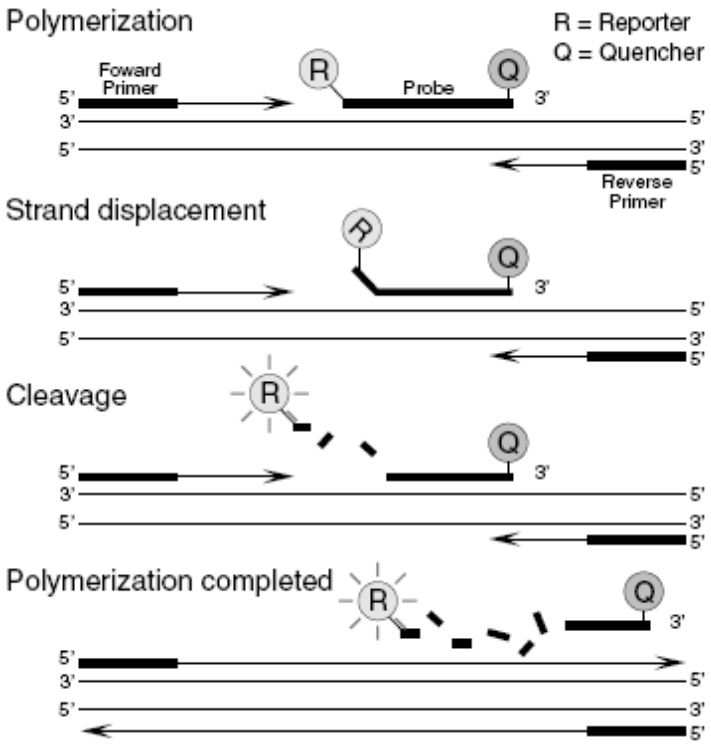


Figure 4.1 (TaqMan Gene Expression assay Protocol) shows the nuclease activity of the Polymerase system

From working dilution plates, 2 µl of DNA was taken from each sample and then transferred into optically clear 384 well plates ordered from Thermo-fast diamond, ABgene. Plates were wrapped in foil paper and left over night on the laboratory bench in order for the samples to dry. The following day a PCR reaction was prepared following standard Applied Biosystems dry DNA protocol.

Plates were sealed with an optically clear "absolute QPCR" adhesive cover ordered from ABGENE and the PCR reaction was carried out in the Applied Biosystems 7900HT Fast Real-Time PCR System machine.

Plates were then subjected to reading using the Applied Biosystems 7900HT Fast Real-Time PCR System. Genotyping calls are made using a clustering algorithm with quality value of 95%. Presence of each allele is marked by colour and position on a generated plot due to level of fluorescence present in each allele. Homozygotes are displayed on extremities of the Y and the X axes whereas heterozygotes are displayed in the middle. Allele discrimination can be visualized in a plot showing the two alleles and the distribution of the three genotypes, the two homozygous and the heterozygous one (Figure 4.2).

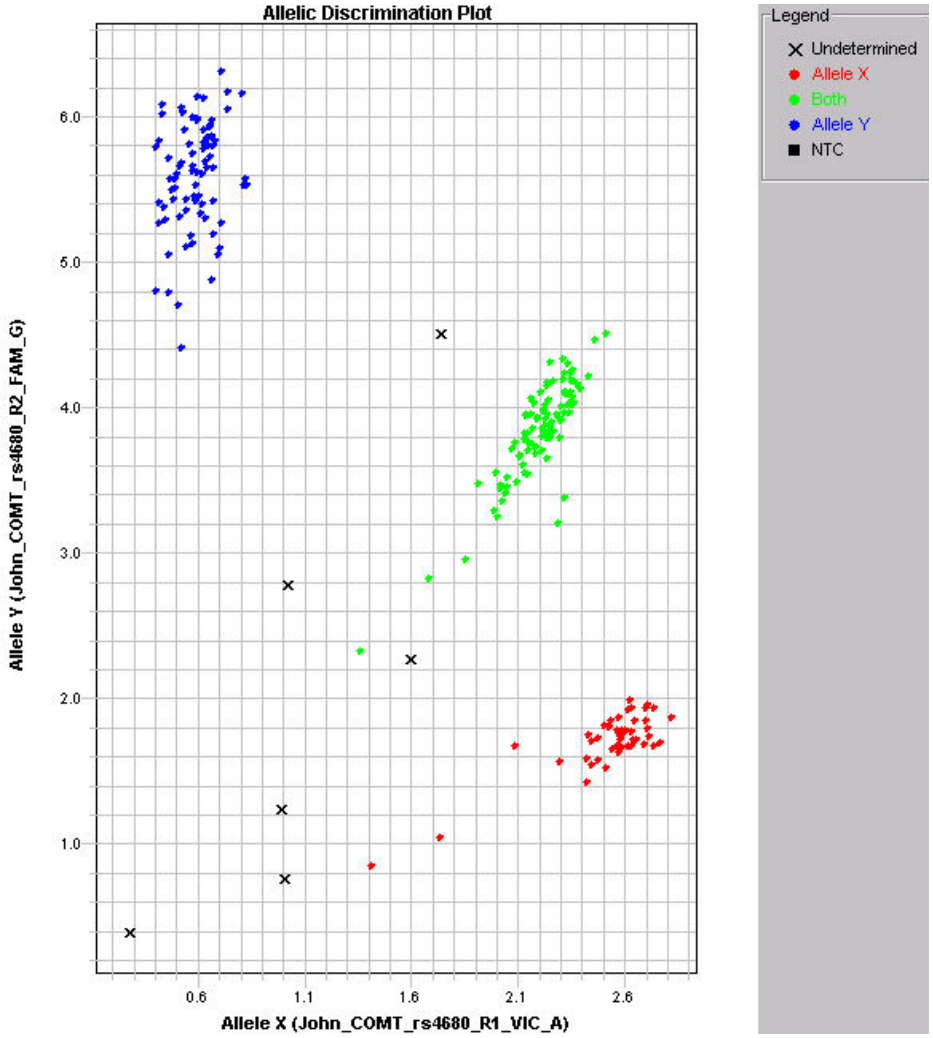


Figure 4.2 shows the allelic discrimination plot resulting from the TaqMan gene expression assay procedure. The two alleles are shown in the axis and in different colours are shown the three different genotypes: blue colour – homozygous G allele - (Val/Val), red colour – homozygous A allele (Met/Met), green colour – Heterozygous -both alleles (Val/Met).

4.5.2 Fragment analysis

Fragment analysis is used to analyse microsatellites markers loci.

Microsatellites are also called Short Tandem Repeats (STRs), Variable Number Tandem Repeats (VNTRs), Short Sequence Repeats (SSRs) and can span between 2 and 7 repeats (Figure 4.3).

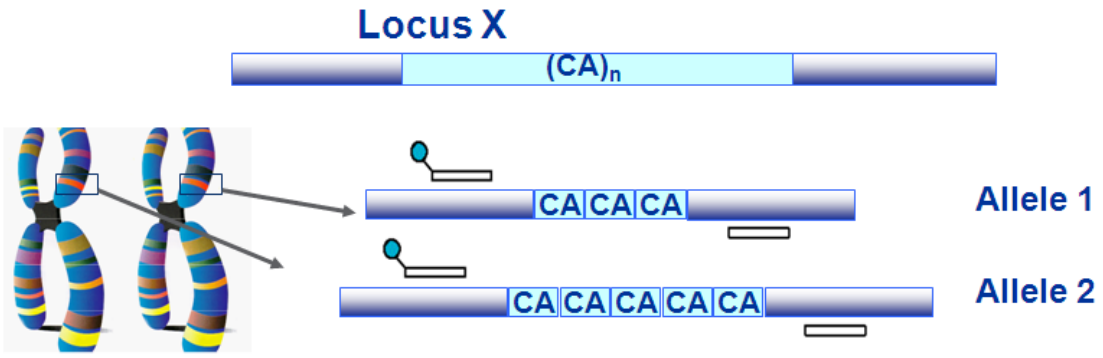


Figure 4.3 Graphic representation of a Microsatellite locus

Microsatellites are amplified by Polymerase Chain Reaction (PCR) with Forward and Reverse primers, one of which is fluorescently labelled. Each sample contains multiple coloured dyes; one of the colours is used as size standard to extrapolate the base-pair sizes of the sample product peaks.

After selecting the primers, 1ul of DNA product is loaded into the working plate and amplified using PCR. In order to achieve good results, each dye is mixed in different ratios to account for differences in fluorescent signal strength. The PCR product is then loaded into a 386 well plate and run in the Applied Biosystems 3130xl Genetic analyzer. Results are given in electropherogram form and analysed using the GeneMapper® software (Figure 4.3).

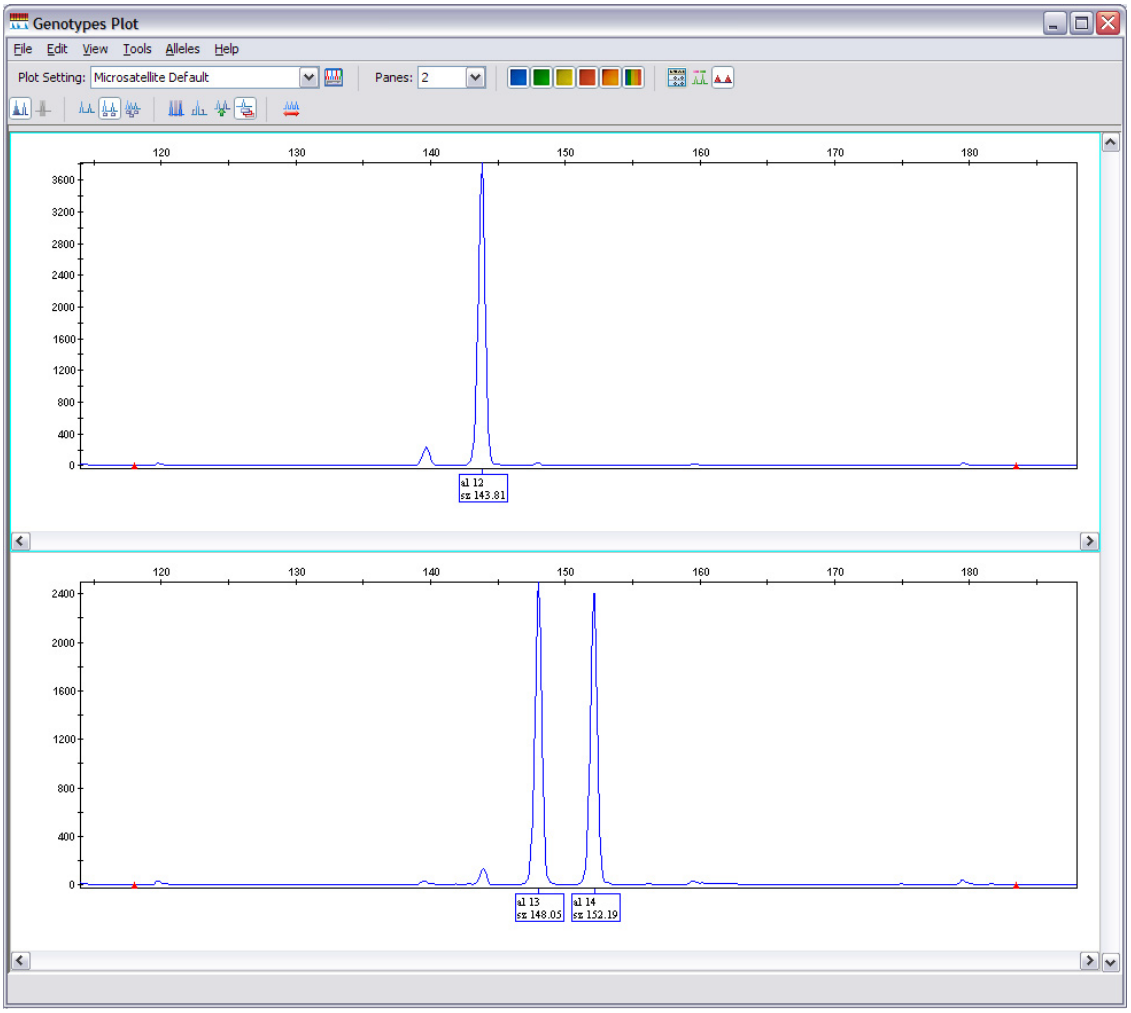


Figure 4.4 Image representing an electropherogram of two different samples analysed with the GeneMapper® software.

4.6 Power calculation

Statistical power calculation was performed using Quanto (Gauderman et al., 2007).

4.6.1 Gene Only

A model assuming a population prevalence of 1% and a log additive effect of an allele was used. For a MAF of 0.1 and odds ratio of 1.2 around 4,000 cases and matched controls are needed for 80% power at a two sided significance of 0.005 (to account for 10 alleles), similar numbers of samples (~6,000) are needed for a MAF of 0.5. For an odds ratio of 2.0, 245 and 121 cases and matched controls are required.

4.6.2 Gene x Environment interaction

A model assuming a population prevalence of 1%, an environmental exposure of 0.4 (in this study cannabis ever use as mean value) and a log additive effect of an allele was used. For a MAF of 0.1 and odds ratio of 1.2 around 65,000 cases and matched controls are needed for 80% power at a two sided significance of 0.005 (to account for 10 alleles), similar numbers of samples (~20,000) are needed for a MAF of 0.5.

The 3 samples analysed in this thesis, as mentioned earlier, consisted of 3 ethnically different populations: the GAP Study sample Caucasian group with 174 cases and 45 controls; the GAP Study sample Black group with 113 cases and 95 controls and the PICOS Study sample with 347 cases and 307 controls. Although the PICOS Study sample is large enough to detect an effect size of 2 in a gene only analysis for an allele frequency of 0.5; replicated true associations in complex disease typically have much smaller odds ratios. This can be considered the case especially for complex disorders like schizophrenia; it is therefore safe to assume that all statistical tests performed in this study are underpowered to detect any true association.

CHAPTER 5

RESULTS

The CNR1 gene and cannabis use in First Episode Psychosis

5.1 Background information

As reviewed and discussed in chapter 3 of this thesis, the endocannabinoid system plays an important role in brain maturation, function and possibly psychopathology. Over the years many studies have focused attention on 2 main areas of interest when analysing the endocannabinoid system, 1. fat metabolism and body weight mechanisms; 2. mental health and drug dependence. The seven transmembrane G coupled endocannabinoid receptor 1, in fact, is widely expressed throughout the brain and particularly abundant in brain regions associated with hunger, appetite and reward (Matias and Di Marzo, 2007) (Wood et al., 2007).

Two CNR1 variants, namely rs6454674, rs806368 and the 2 haplotypes containing genotypes of them, have been reported to increase risk for cocaine dependence in 3 separate study samples. (Zuo et al., 2009). Furthermore, rs806368 was also associated with cocaine induced paranoia in the same study (Zuo et al., 2009). Two other polymorphisms were associated with cocaine induced paranoia in the same study, rs1049353 and rs2146274 in a family based cohort (Zuo et al., 2009). More recent findings come from Marcos et al., who found a haplotype containing rs6454674, rs1049353 and rs806368 to be associated with alcohol dependence, with positive interaction also at the alleles of the last two polymorphisms of the haplotype (Marcos et al., 2012). A meta-analysis performed by Benyamina et al., however, only found only a marginal effect of the AAT repeat within the CNR1 gene and drug dependence (Benyamina et al., 2011). Finally Bienertova-Vaskus et al., found an effect of the CNR1 haplotype made up of rs6454674, rs1049353 and rs806368 on self reported number of cigarettes smoked (Bienertova-Vaskus et al., 2012). These finding were a part of a study on metabolic response and feeding control (Bienertova-Vaskus et al., 2012).

Studies on the association between the CNR1 gene and metabolic measures also show positive association of various polymorphisms, with some overlapping markers. Tiwari et al., reported a polymorphism, rs806378, to be associated with weight gain in patients treated with olanzapine and clozapine, thus highlighting once again, the close relationship between the endocannabinoid system and the metabolic system (Tiwari et al., 2010). Park et al., however, found no significance between variation at 3 CNR1 loci (rs1049353, rs806368, and rs4707436) and olanzapine induced weight gain (Park et al., 2011). In a study of 2411 participants, rs806365 was found to be associated with Type 2 Diabetes, Coronary Heart Disease and insulin resistance (De Miguel-Yanes et al., 2011).

On the same line, findings of Jaeger et al., found a marginal association of the rs1049353-rs12720071-rs806368 haplotype on waist to hip ratio, though association was not significant after multiple testing correction (Jaeger et al., 2009). In the same study, no association was found between rs104973, rs12720071 and measures of central obesity; only rs806368 returned significant p-value even after multiple testing correction on WTHR. (Jaeger et al, 2009). Other polymorphisms, namely rs6928499, rs1535255, and rs2023239 were also found to be associated with lower risk of metabolic syndrome in schizophrenic patients (Yu et al., 2013), whereas, in the same study, some others, namely rs806377, rs1049353, rs6454674, and rs806379 returned no positive results (Yu et al., 2013). Furthermore, rs1049353 has returned interesting results in 2 other studies, with A allele associated to a lack of improvement of leptin levels in obese subjects exposed to both high monounsaturated and polyunsaturated diet (De Luis et al., 2013) and the G allele associated to antipsychotic refractoriness but not psychosis (Hamdani et al., 2009). Indeed, rs806374 was found to predispose schizophrenic patients to Tardive Diskinesia, after treatment with antipsychotics (Tiwari et al., 2012).

Several recent studies have also implicated markers at the CNR1 gene in anxiety, schizophrenia and other disorders. rs2180619 was found to have an additive effect on anxiety extinction in healthy subjects whereas rs1049353 had no effect (Heitland et al., 2012). The haplotype rs806368-rs1049353-rs806371 was found to be associated with Major Depression, increasing significance after stratification for Melancholia and psychotic symptoms (Mitjans et al., 2013). Furthermore, rs806371 and rs806368 were found to be associated with Major depression, Melancholia and increased risk of non remission; there was also some evidence of a better response in rs806368 C allele carriers (Mitjans et al, 2013). Finally, rs1049353, rs7766029 and rs806366 were shown to increase risk of psychosis on a cohort of 150 schizophrenic patients and 350 healthy controls, even though not retaining significance after multiple testing correction (Costa et al., 2013). Fewer studies have reported associations with polymorphisms within the CNR1 gene and cannabis use, Schacht et al., reported rs2023239 to have a group by genotype interaction, where the G allele predicted lower volume of bilateral hippocampi in cannabis users (Schacht et al., 2012). Onwameze et al., suggested a diplotype effect of rs12720071 combined with rs12199654 in the MAPK14, interacting with cannabis use in white matter brain volumes (Onwameze et al., 2013). In summary, several polymorphisms within the CNR1 gene have been investigated in relation to metabolic changes in the normal population as well as in anti psychotic treated schizophrenia patients with discordant results. In the same way, results differ within studies on drug dependence like cocaine or cannabis use and even more on main effect on psychosis. This could be related to the strength of the associations. As GWA Studies teach us, to achieve meaningfull results of statistical true relevance, samples need to be well characterised and in the range of thousands. These findings could therefore

be the consequence of false associations, which give the picture of heterogeneity of results whereas it could be a consequence of poor statistical power.

There appear to be polymorphisms more widely studied than others, like, for example rs1049353, also included in the set of tag SNPs analysed in this thesis. It has been shown to return positive associations with cocaine induced paranoia (Zuo et al., 2009), lack of improvement in leptin levels (De Luis et al. 2012) and treatment resistance in psychotic patients (Hamdani et al., 2008); or as part of a 3 SNPs haplotype rs806368-rs1049353-rs806371 with Major Depression (Mitjans et al., 2013), rs6454674, rs1049353 and rs806368 on self reported number of cigarettes smoked (Bienertova-Vaskus et al., 2012), rs1049353-rs806368 with alcohol dependence (Marcos et al., 2010) with both alleles seemingly involved. This suggests a potential role in metabolism and/or drug abuse and even on psychosis (Costa et al., 2013). The polymorphism rs806368 has also been reported by several studies, as associated SNP or as part of a haplotype. This marker, as well as several others, reported by recent studies, has not been included in the selection of SNPs to be analysed in this thesis. In light of these recent contrasting, but interesting results, it would have been reasonable to include previously reported markers for the analysis, or even proceed with sequencing of the entire gene. SNPs selection, however, was performed in 2007, when the CNR1 gene was not widely studied and the all above mentioned studies were not yet available. I therefore, based on HapMap data available in 2007, tagged the whole length of the CNR1 gene in an attempt of getting the main signals from independent SNPs. By tagging the whole length of the gene, I choose not to start with a hypothesis a priori driven experiment. SNPs analysed in my thesis are aimed to give complete coverage of the gene which was achieved. Table 5.1 shows the selection of SNPs tagged and analysed in this thesis and whether they have been reported, with or without significance in previous studies in metabolism (with special attention to anti psychotic weight gain) and mental health.

rs806365	T2D and CHD De Miguel-Yanes et al., 2011)											
rs806366	Main effect on schizophrenia (Costa et al., 2013)											
rs1049353	CIP (Zuo et al., 2009)	Haplotype effect on AD (Marcos et al., 2012)	Haplotype effect on self reported cigarette intake (Bienertova-Vaskus et al., 2012)	No association found on olanzapine induced weight gain (Park et al., 2011)	No association with drug abuse (Benyamin a et al., 2011)	No association found with measure of central weight gain in Brazilian population (Jaeger et al., 2009)	Lack of improvement in leptin levels (De Luis et al., 2012)	No effect in anxiety extintion (Heitland et al., 2012)	Haplotype effect on MD (Mitjans et al., 2013)	Main effect on schizophrenia (Costa et al., 2013)	No association found with metabolic syndrome ins schizophrenics patients (Yu et al., 2013)	Treatment resistance with atypical antipsychotics (Hamdani et al., 2008)
rs806371	Allelic and haplotype effect on MD											

	and Melancholia (Mitjans et al., 2013)											
rs806374	TD in schizophrenic patients treated with antipsychotic (Tiwari et al., 2012)											
rs12195101	No association with measure of central weight gain in Brazilian population (Jaeger et al., 2009)											
rs806375												
rs806377	No significance	No association										

	in aetiology of MD/citolapram response (Mitjans et al., 2013)	found with metabolic syndrome ins chizophrenic patients (Yu et al., 2013)										
rs806378	Weight gain in olanzapine and clozapine patients Tiwari et al., 2010)											

Table 5.1: Summary of recent studies reporting significant and non significant association of the SNPs analysed in this thesis and different disorders

5.2 Hypothesis under investigation

In this chapter I will analyze the potential contribution of 15 polymorphisms, namely rs10485171, rs806365, rs806366, rs12189668, rs1049353, rs806369, rs806371, rs806374, rs12195101, rs806375, rs806377, rs806378, rs2023239, rs1535355, rs6454672 within the CNR1 gene to the increase in risk for psychosis.

I hypothesise that genetic polymorphisms analysed will give an increase in risk for psychosis.

I will then run an exploratory analysis to check for haplotypic association with psychosis. Haplotypes are analysed with window of 3.

Finally, I will analyse Gene x Environment interaction between rs1049353 and lifetime cannabis use.

I hypothesise that the multiplicative effect of lifetime cannabis use and rs1049353 gives an increase in risk for psychosis.

5.3 SNPs selection

Markers of the CNR 1 gene have been selected using the international HapMap data and SNPs within the CNR1 gene are presented graphically in a linkage disequilibrium plot, so that only a subset of them needs to be genotyped (Figure 5.1).

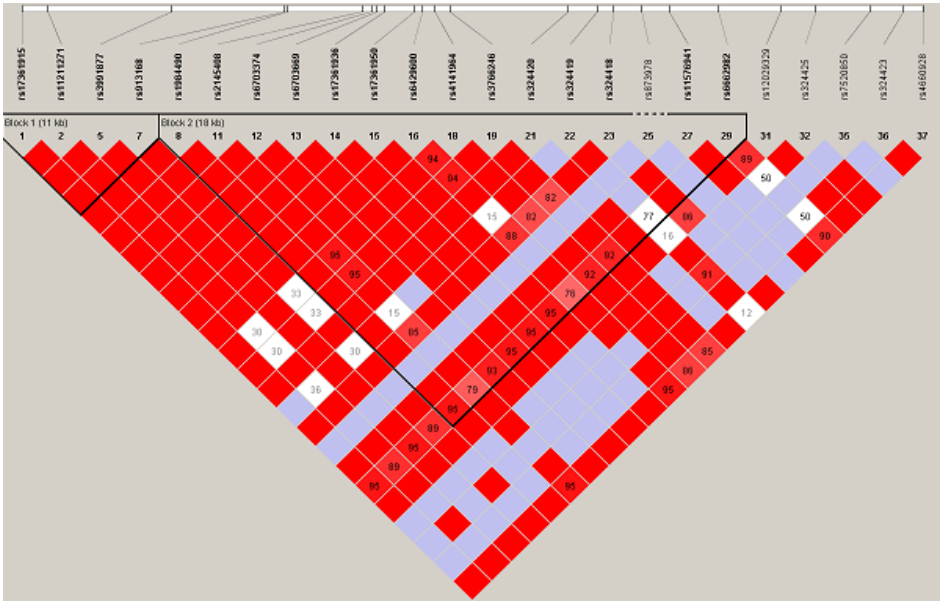


Figure 5.1 shows a linkage disequilibrium plot generated with Haploview for the CNR1 gene based on genotyping data available from the HapMap project (The International HapMap Consortium, 2005).

After choosing the gene, CNR1 in this study, the HapMap web page produces a diagram of the gene with all SNPs present in the exact area under investigation. Figure below shows the output of the webpage given after selection of the CNR1 gene (Figure 5.2)

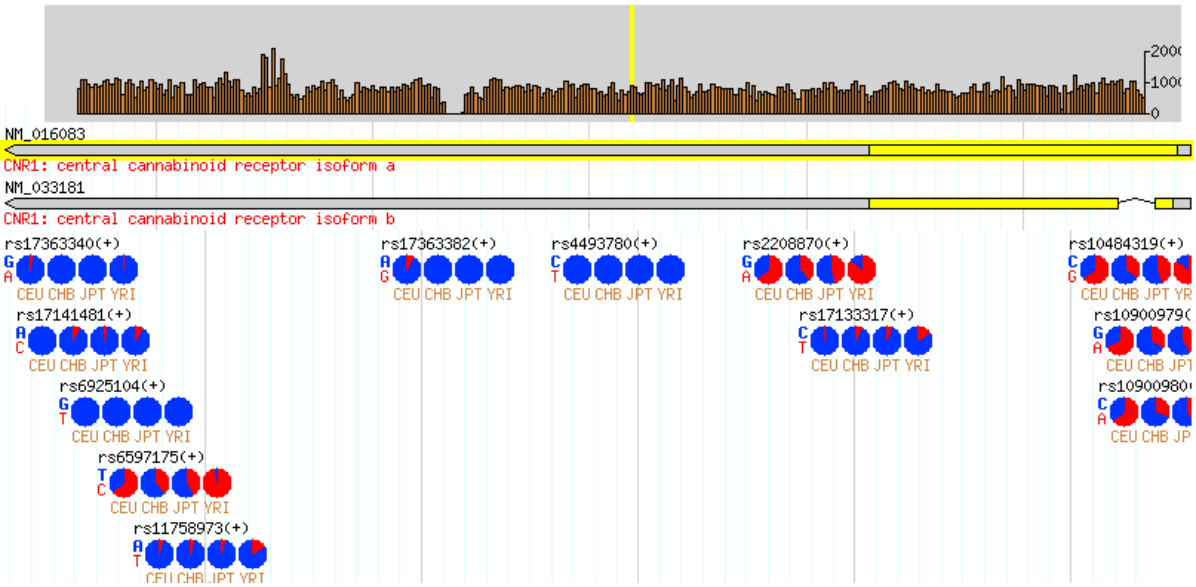


Figure 5.2 shows data obtainable from HapMap. In this case the gene illustrated is the CNR1 gene with several of the validated SNPs based on genotyping data available from the HapMap project (The International HapMap Consortium, 2005)

The GAP Black study population is West African and Afro Caribbean and it is genetically very close to the Yoruba population. When tagging SNPs it was decided to choose Yoruba and CEPH populations.

Tag SNPs have been selected using the Haploview program with HW p-value cutoff=0.0010; min genotype=75%; minimum minor allele frequency=0.0010; r2 threshold 0.8; LOD threshold for multi-marker tests=3.0. Aggressive tagger with two or three marker haplotype was used. Non synonymous SNPs have been force included because of their importance in association studies. A list of SNPs and their chromosomal position can be seen in table 5.2.

Table 5.2 List of markers selected via Haploview within the CNR1 gene

Gene	SNP	Chr position	MAF *	Sequence
CNR1	rs10485171	89261282	G=0.329	CAAAGACAGAGCAATCATCTGGAAAA**[C/T]**GGGAGCTAAAAACACAAGCTTTTGT
CNR1	rs806365	89263841	T=0.397	TTCATAGCTAGCTCTTACTTTGTCTT**[C/T]**AGTGCTCTATTCAAGCATCACCTCT
CNR1	rs806366	89265481	C=0.415	ACACACTTCTACATTTCCGCTACAAG**[C/T]**GGGTCTGCATCTACACAGCGGTAAT
CNR1	rs12189668	89267257	C=0.001	AGTTAAAATCTTTAATGAAATAATGC**[C/T]**TTTTAAAGGTGTGGTGGCCTTTTCT
CNR1	rs1049353	89271527	T=0.137	GCCGCAGAAAGCTGCATCAAGAGCAC**[A/G]**GTCAAGATTGCCAAGGTAACCATGT
CNR1	rs806369	89274070	T=0.305	ATGGAGGAGGCCTCCTGATAGTCCCC**[C/T]**TCATGAGCAGGTTGGTGACACAAGT
CNR1	rs806371	89274255	G=0.278	GAGAACTGATCTTACTATTTATAAAT**[G/T]**TTTGTTTAAAATGTGGGCTATTCAT
CNR1	rs806374	89275212	C=0.402	AAGTAATTTGGAACAGGCATAAAGTG**[C/T]**TAAATGTGGCCCACCTTCCCACATT
CNR1	rs12195101	89275717	G=0.002	TGCCACTTGGGCTACACCAGATGAAT**[G/T]**TTAGTTCCATAAACACAGCAAATTG
CNR1	rs806375	89276413	T=0.373	TGGTGAACAGAGAAAGAGCCCTCAGC**[A/T]**CTAATGTGACAGGTAAGCCAGAAGG
CNR1	rs806377	89276615	C=0.478	GGCTTCTGAACCAGTTCTGCACACCT**[C/T]**TCCTGCAACTGTCATAGAATAAAGC
CNR1	rs806378	89277443	T=0.182	TCCCTCTATTACAGGCCTCATCACGT**[C/T]**GTATAATCAGGAGTTCACATATTTA
CNR1	rs2023239	89278374	C=0.167	CTAGGTTTGTGGATGTGCCAGGACCA**[C/T]**GTAAGGAACAGCTCTCTCATATATT
CNR1	rs1535255	89279100	G=0.168	TAAGCCTCAGTATTTCATCTGTAAAA**[G/T]**GGGGATGAGGATGATGACAATAATG
CNR1	rs6454672	89279462	C=0.141	GTTATATCCAGCCATTTGAGAAACTT**[C/T]**ACATATATTCTATCATTAAATTGTC

*MAF= Minor Allele Frequency as reported in NCBI search engine

5.4 SNPs Genotyping

All samples of the Genetics and Psychosis research Study (GAP) and all samples of the PICOS research Study were extracted, diluted, prepared in 186 well plates and shipped to Prevention Genetics for genotyping. For detailed explanation of study sample characteristics, procedures on DNA extraction and sample preparation please see chapter 4 of this thesis (Materials and Methods).

5.5 Statistical Analysis

In this chapter samples analysed are from the GAP Study (both Caucasian and Black population included) and from the PICOS study.

The GAP Study Caucasian population analysed consisted of 174 psychotic patients and 45 non psychotic subjects;

The GAP Study Black population analysed consisted of 113 psychotic patients and 95 non psychotic subjects;

The PICOS Study sample consisted of 347 psychotic patients and 307 non psychotic patients.

The three main groups of samples were tested separately: Caucasian group (GAP); Black African group (GAP); Caucasian Italian group (PICOS).

5.5.1 Statistical tests performed

With the three sets of samples, namely the GAP study sample (including Caucasian subjects), the GAP study (including the black population) and the PICOS study sample (consisting of Caucasian population) the following statistical tests were performed:

- Hardy Weinberg Equilibrium Test
- Logistic regression test for allelic and genotype association analysis between each SNP and disease status
- Haplotype association analysis with sliding window of 3
- Haplotype Conditional test for independence effect of rs1049353
- Bonferroni multiple testing correction
- Logistic regression test for the Gene x Environment analysis (only the GAP Caucasian and Black study sample)

5.5.2 Hardy Weinberg Equilibrium Test

Hardy Weinberg Equilibrium Test was performed using PLINK software for genetic analysis throughout the thesis (Purcell et al., 2007).

The command used for the HWE test is --hardy

The full line of command used to run the HWE test in this thesis is:

Plink --file mydata --hardy

A file containing three entries for each SNP was then generated and saved as plink.hwe

The file contains calculations of the HWE test for all, affected only or unaffected only subjects.

In this thesis, only results of the unaffected group of subjects are discussed and reported in the tables.

5.5.3 Allelic and genotype association analysis

Statistical analysis carried out to establish association between allelic and genotype variation at each locus within the CNR1 and psychosis was performed using PLINK software for genetic analysis (Purcell et al., 2007).

The main effect of each SNP on psychosis was tested with logistic regression. PLINK calculates a basic association of a disease trait by comparing allele frequencies between cases and controls (Purcell et al., 2007). The association commands used are --assoc --logistic.

SNPs alleles and genotypes were treated as independent variables and disease outcome (psychosis) was treated as dependent variable. It was coded in the program as a binary disease status: 1=case 0=control. Because the disease outcome was a binary value, --1 was added to the command line used.

The complete set of commands used to calculate association in this thesis was:

Plink –file mydata --1 --assoc --logistic

A file containing both allelic and genotype association calculation was then created and saved as plink.assoc.

5.5.4 Haplotype analysis

Linkage Disequilibrium between SNPs within the CNR1 gene was computed using Haploview version 3.32 (Barrett et al., 2005). All markers were within 500Kb which is the default value for distance.

A graphical representation of linkage disequilibrium between markers was created with LD-Plus, a program that is freely available via a web interface (https://chgr.mc.vanderbilt.edu/ldplus). D' and r2 values, were calculated using Haploview (Barrett et al., 2005).

Haplotype analysis was then carried out using PLINK software for genetic analysis (Purcell et al., 2007).

In order to phase a set of SNPs for haplotypic analysis, both the --chap and the --hap--snp were used together.

--mhf 0.05 was used to increase the minimum haplotype frequency

--window 3

The full line of command used in this thesis for haplotype analysis is:

Plink --bfile mydata --1 --hap-window 3 --hap-assoc

The output file generated by the program after this calculation is saved as plink.hap

After some haplotypes returned significant p-value, I proceeded to a conditional test to check whether there was an independent of rs1049353. This SNP showed significant association with psychosis in the GAP study sample with Caucasian subjects (corrected p-value=0.03). The conditional test was run to check for independence of rs1049353 from haplotypic effects formed by the remaining SNPs.

The full line of command used was

Plink --file mydata --hap--snps rs1048517-rs6454672 --chap --independent-effect rs1049353

The output file reports Chi-square, df and p-values.

5.5.5 Gene x Environment analysis

GxE analysis was performed with logistic regression test using HapStat version 3.0 (Lin et al., 2008). Hapstat is a user-friendly software that allows testing of single markers and haplotype-disease association. The haplotype association is calculated by maximizing the observed data likelihood that accounts for phase uncertainty and study design (Lin et al., 2008).

Environmental variable entered was:

- cannabis use (variable modeled as binary trait: yes/no answer)

Genetic variables entered were:

- rs1049353

5.5.6 Bonferroni Correction for multiple testing

Bonferroni conservative correction for multiple testing was applied after allelic and genotype association analysis and after haplotype analysis was performed.

For the logistic regression, the markers analysed were 13, Bonferroni corrected p-value calculated as 0.05/15 would be 0.003.

When Bonferroni was applied to correct for the number of sliding windows generated with haplotype analysis, there were a total of 15 markers and 13 tests performed. Bonferroni corrected p-value calculated as 0.05/13 would be 0.003.

In the tables, Bonferroni corrected p-value is reported as χ^2 p-value * number of tests performed.

5.6 Results

5.6.1 Hardy Weinberg Equilibrium Test

Hardy Weinberg Equilibrium was examined in non psychotic control groups of both the GAP Study and the PICOS Study separately. As mentioned earlier, the GAP Study is made of two main ethnic groups: the Caucasian and the Black group. In every statistical analysis performed in this thesis, they were analysed separately as 3 distinct populations. A HWE test was therefore performed for each group: Caucasian, Black and PICOS. The GAP Study Black population included participants from Black African, Black Caribbean origins as well as any other ethnicity mixed with black Caribbean or Black African. Deviation from equilibrium was checked for a total of 15 SNPs namely rs10485171, rs806365, rs806366, rs12189668, rs1049353, rs806369, rs806371, rs806374, rs12195101, rs806375, rs806377, rs806378, rs2023239, rs1535355, rs6454672 within the CNR1 gene.

None of the markers failed the HWE test after accounting for multiple testing correction (0.05/15=0.003) in the GAP Black and Caucasian non psychotic groups (Complementary Tables C.1 and C.2).

In the PICOS Study samples, 15 SNPs were tested with the Hardy Weinberg Equilibrium test: rs10485171, rs806365, rs806366, rs12189668, rs1049353, rs806369, rs806371, rs806374, rs12195101, rs806375, rs806377, rs806378, rs2023239, rs1535355, rs6454672 within the CNR1 gene. Analysis confirmed that all SNPs in the non psychotic PICOS Study control group were in equilibrium (Complementary Table C.3).

5.6.2 Association between allelic and genotype variation at each locus within the CNR1 gene and Psychosis

The main effect of each SNP at each locus within the CNR1 gene on psychosis was tested with a χ^2 test in the 3 study samples separately.

In the GAP Caucasian study sample 2 markers, namely rs806378 and rs1049353 returned higher significance values, p-value 0.004 and p-value 0.002 respectively. Significance was only retained by 1 marker rs1049353 with a p-value of 0.03 (0.002*15), for which the C allele seems to be over expressed in psychotic participants. Bonferroni set threshold for significance for this set of test would be p-value=0.003 (Table 5.2).

The higher values, however, probably relate to low MAFs at both loci in the population examined. The MAFs in the affected and non affected population are 0.12 and 0.26, 0.04 and 0.14, respectively; as it can be seen in table 4.5. I therefore had a very low statistical power to detect any true association as explained in detail later in the conclusions paragraph of this chapter.

In the GAP Black group no association was found (table 5.3).

Genotype variation at the two loci did not show association with psychosis (Complementary Table C.4 and C.5).

In the PICOS sample allelic variation was found to be associated with psychosis at rs806378 locus (p-value=0.03 OR=0.77) and at rs806371 locus (p-value=0.047 OR=1.34). We also have to bear in mind, in this study sample, that 13 tests were performed, therefore the p-value reported are not adjusted for multiple testing. If we were, however, to adjust for multiple testing using the conservative Bonferroni, none of the observed p-value would have met criteria for significance (Bonferroni adjusted p-value= 0.003) (Table 5.4).

Genotype variation however did not show any significance at any of the loci (Complementary Table C.6).

The MAFs of the affected and unaffected populations within the PICOS sample are low: 0.23 and 0.28 respectively. It is thus possible that the higher value observed is due to a statistical false positive. I had, in fact, very low power to detect a true effect of a locus with a low MAF. The number of case-control pair should range in the thousand. This is further reviewed in the final paragraph of this chapter. Results are summarised in table 5.5.

TABLE 5.3: Association between allelic variation at each locus within CNR1 and psychosis in the GAP Caucasian sample

Gene	rs number	Minor Allele/ Other allele	psychotic participants (MAF)	psychotic participants (N)	non psychotic participants (MAF)	non psychotic participants (N)[(a)]	CHISQ (DF=1)*	P-Value/Corrected p-value	OR
CNR1	rs10485171	C/T	0.43	153	0.50	35	1.19	0.275	0.75
CNR1	rs806365	T/C	0.32	157	0.34	32	0.20	0.656	0.88
CNR1	rs806366	C/T	0.27	152	0.39	35	3.71	0.054	0.59
CNR1	rs12189668	C/T	0.01	156	0.04	35	2.87	0.090	0.29
CNR1	rs1049353	A/G	0.04	156	0.14	35	9.32	**0.002/0.03**	0.28
CNR1	rs806369	T/C	0.11	154	0.17	35	1.75	0.186	0.62
CNR1	rs806371	G/T	0.29	157	0.22	32	1.23	0.268	1.44
CNR1	rs806374	C/T	0.43	159	0.35	31	1.13	0.287	1.36
CNR1	rs12195101	G/T	0.04	157	0.06	35	0.34	0.563	0.71
CNR1	rs806375	A/T	0.45	155	0.43	34	0.14	0.706	1.11
CNR1	rs806377	T/C	0.35	156	0.40	34	0.48	0.489	0.83
CNR1	rs806378	C/T	0.12	155	0.26	35	8.23	**0.004/0.06**	0.40
CNR1	rs2023239	C/T	0.36	157	0.34	35	0.07	0.788	1.08
CNR1	rs1535255	G/T	0.32	159	0.24	35	1.52	0.218	1.45
CNR1	rs6454672	C/T	0.31	154	0.23	35	1.62	0.203	1.48

MAF= Minor allele frequency

OR=Odd Ratio

(a)= number of participants (Psychotic and non psychotic) for genotype groups at each locus

* Degrees of freedom=1

TABLE 5.4: Association between allelic variation at each locus within CNR1 and psychosis in the GAP Black sample

Gene	rs number	Minor Allele/ Other allele	psychotic participants (MAF)	psychotic participants (N)	non psychotic participants (MAF)	non psychotic participants (N)[(a)]	CHISQ (DF=1)*	P-Value	OR
CNR1	rs10485171	C/T	0.40	97	0.42	71	0.06	0.805	0.95
CNR1	rs806365	T/C	0.43	96	0.37	71	1.48	0.224	1.32
CNR1	rs806366	T/C	0.52	97	0.44	70	2.24	0.135	1.40
CNR1	rs12189668	C/T	0.05	101	0.07	73	0.29	0.587	0.78
CNR1	rs1049353	A/G	0.28	99	0.20	75	3.15	0.076	1.58
CNR1	rs806369	T/C	0.26	95	0.19	70	2.73	0.099	1.57
CNR1	rs806371	G/T	0.14	98	0.21	74	2.63	0.105	0.63
CNR1	rs806374	C/T	0.36	96	0.37	74	0.00	0.972	0.99
CNR1	rs12195101	G/T	0.03	100	0.05	74	0.33	0.564	0.73
CNR1	rs806375	T/A	0.48	96	0.51	71	0.29	0.591	0.89
CNR1	rs806377	C/T	0.45	99	0.53	74	1.87	0.172	0.74
CNR1	rs806378	T/C	0.28	96	0.29	75	0.08	0.780	1.07
CNR1	rs2023239	C/T	0.17	99	0.28	72	5.62	0.018	0.54
CNR1	rs1535255	G/T	0.14	101	0.22	72	3.02	0.082	0.61
CNR1	rs6454672	C/T	0.12	99	0.16	71	1.15	0.283	0.71

MAF= Minor allele frequency

OR=Odd Ratio

(a)= number of participants (Psychotic and non psychotic) for genotype groups at each locus

* Degrees of freedom=1

TABLE 5.5: Association between allelic variation at each locus within CNR1 and psychosis in the PICOS Study

Gene	rs number	Minor Allele/ Other allele	psychotic participants (MAF)	psychotic participants (N)	non psychotic participants (MAF)	non psychotic participants (N)[(a)]	CHISQ (DF=1)*	P-Value/Corrected p-value	OR
CNR1	rs10485171	T/C	0.38	266	0.39	505	0.26	0.613	0.95
CNR1	rs806365	T/C	0.47	273	0.49	510	0.45	0.502	0.93
CNR1	rs806366	T/C	0.48	263	0.45	504	1.94	0.062	1.19
CNR1	rs12189668	T/C	0.00	273	0.00	510	1.87	0.172	NA
CNR1	rs1049353	G/A	0.23	267	0.22	502	0.20	0.652	1.06
CNR1	rs806371	G/T	0.17	275	0.13	504	3.96	**0.047/0.6**	1.34
CNR1	rs806369	T/C	0.34	270	0.35	509	0.03	0.869	0.98
CNR1	rs806374	C/T	0.34	265	0.33	507	0.23	0.632	1.06
CNR1	rs12195101	G/T	0.00	270	0.00	514	1.05	0.305	0.00
CNR1	rs806375	A/T	0.38	264	0.43	501	3.50	0.061	0.81
CNR1	rs806377	C/T	0.48	266	0.50	502	0.55	0.458	0.92
CNR1	rs806378	C/T	0.23	269	0.28	507	4.46	**0.035/0.45**	0.77
CNR1	rs2023239	T/C	0.18	268	0.17	510	0.89	0.345	1.14
CNR1	rs1535255	G/T	0.18	267	0.17	504	0.63	0.428	1.12
CNR1	rs6454672	C/T	0.14	271	0.11	511	1.76	0.185	1.24

MAF= Minor allele frequency

OR=Odd Ratio

(a)= number of participants (Psychotic and non psychotic) for genotype groups at each locus

* Degrees of freedom=1

TABLE 5.6 Summary of non corrected VS corrected p-values obtained with the allelic test across all population examined

SNPs	GAP samples (Caucasian group)	GAP samples (Black group)	PICOS samples
rs1049353	Cases(N)=152 Controls(N)=35 p-value=**0.002/0.03** MAF AFF=0.04 MAF UN=0.14	Cases(N)=97 Controls(N)=70 p-value=0.13 MAF AFF=0.28 MAF UN=0.20	Cases(N)=263 Controls(N)=504 p-value=0.062 MAF AFF=0.23 MAF UN=0.22
rs806378	Cases(N)=155 Controls(N)=35 p-value=**0.004/0.05** MAF AFF=0.12 MAF UN=0.26	Cases(N)=96 Controls(N)=75 p-value=0.78 MAF AFF=0.28 MAF UN=0.29	Cases(N)=269 Controls(N)=507 p-value=**0.035/0.45** MAF AFF=0.23 MAF UN=0.28
rs806371	Cases(N)=157 Controls(N)=32 p-value= 0.2 MAF AFF= 0.2 MAF UN= 0.22	Cases(N)=98 Controls(N)=74 p-value=0.1 MAF AFF= 0.14 MAF UN= 0.21	Cases(N)= 275 Controls(N)= 504 p-value= **0.05/0.65** MAF AFF= 0.17 MAF UN= 0.13

OR= Odds Ratio

MAF AFF=Minor Allele Frequency in affected population

MAF UN=Minor Allele Frequency in unaffected population

The above table shows a summary of markers that have returned a significant non corrected p-value from the χ^2 test. The point of the summary is to show the same markers with non corrected VS corrected p-value across the 3 different samples analysed in this chapter. As it can be seen rs1049353 shows association with psychosis only in the GAP study sample with Caucasian subjects. rs806371 shows association only in the PICOS Study samples, but changed to non significant after multiple testing correction. rs806378 shows association with psychosis in the GAP Caucasian Study sample and the PICOS Study sample but retained significance only in the GAP Caucasian Study sample. All the above mentioned significant p-values, however, are probably due to low MAFs that can be observed across the 3 study sample.

5.6.3 Gene x Environment analysis

As shown above in paragraph 5.6.2, rs1049353 has been found to have a main effect on psychosis in the Caucasian population. In order to explore the correlation between rs1049353 and cannabis use, I performed a logistic regression test in both the Caucasian and the Black samples.

There was no association between rs1049353 and cannabis use in either of the 2 study samples (Tables 5.7). rs1049353 did not seem to have any effect on cannabis use.

Table 5.7 Correlation between cannabis use and rs1049353

POPULATION	GENE	MARKER	P-VALUE	STANDARD ERROR	Z SCORE
Caucasian	CNR1	rs1049353	0.5	0.2	-1.3
Black	CNR1	rs1049353	0.1	0.2	0.5

Gene x Environment interaction was calculated with logistic regression test under additive model. No interaction was found between rs1049353 and cannabis use in either of the GAP Study sample groups (Table 5.8).

Table 5.8 Gene x Environment interaction between rs1049353 and Cannabis use

POPULATION	GENE	MARKER	P-VALUE	STANDARD ERROR	Z SCORE
Caucasian	CNR1	rs1049353	0.32	0.25	1.2
Caucasian	CNR1	rs1049353	0.5	0.3	-0.5

5.6.4 Haplotype analysis

I decided to further explore the data by performing haplotype analysis as it may reveal additional associations. The level of Linkage Disequilibrium (LD) for the SNPs within the CNR1 gene was assessed using haploview (Barrett et al., 2005) and haplotypic association analysis was carried out using PLINK version 1.7 (Purcell et al., 2007).

For a better understanding of the level of correlation between the SNPs in the population examined in this chapter, I calculated the D' and the r2 values with haploview (Table 5.9, Table 5.10 and Table 5.11). They are also displayed in figure 5.3, 5.4 and 5.5 representing LD blocks plots with D' and r2 values.

Figure 5.3: Linkage Disequilibrium Plot computed with markers within the CNR1 gene in the GAP Caucasian sample

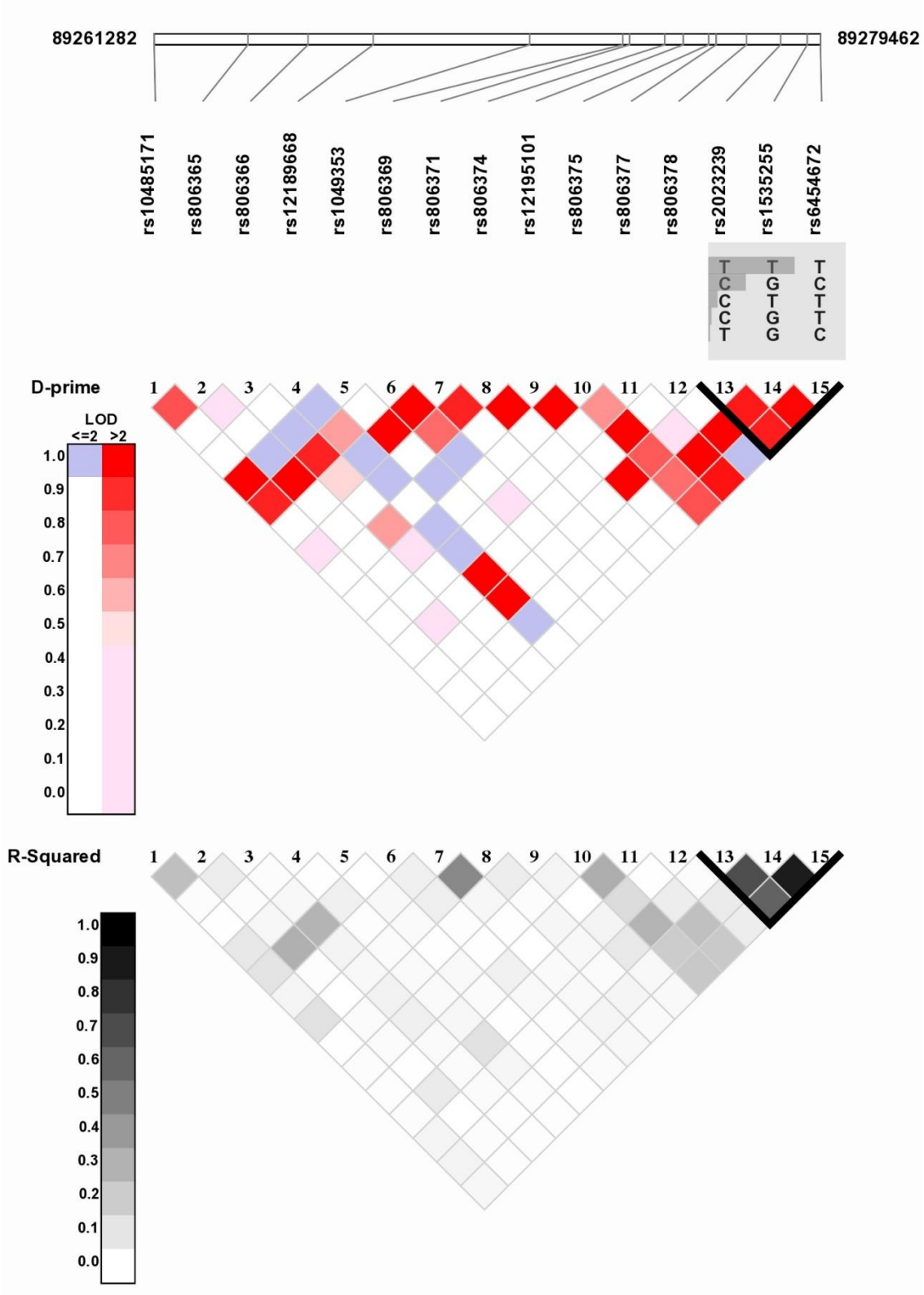


Table 5.9 D' and r2 values calculated with Haploview in the GAP Caucasian sample (shown in bold markers with higher D' and r2 scores further discussed in the conclusion paragraph of this chapter)

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs10485171	rs806365	0.817	0.248
rs10485171	rs806366	0.177	0.01
rs10485171	rs1049353	1	0.089
rs10485171	rs806371	0.369	0.039
rs10485171	rs806374	0.449	0.113
rs10485171	rs806375	0.116	0.008
rs10485171	rs806377	0.082	0.005
rs10485171	rs806378	0.271	0.01
rs10485171	rs2023239	0.231	0.038
rs10485171	rs1535255	0.279	0.043
rs10485171	rs6454672	0.246	0.031
rs806365	rs806366	0.285	0.069
rs806365	rs1049353	1	0.029
rs806365	rs806371	0.144	0.004
rs806365	rs806374	0.009	0
rs806365	rs806375	0.195	0.014
rs806365	rs806377	0.056	0.003
rs806365	rs806378	0.458	0.078
rs806365	rs2023239	0.04	0
rs806365	rs1535255	0.222	0.01
rs806365	rs6454672	0.196	0.007
rs806366	rs1049353	1	0.027
rs806366	rs806371	0.516	0.041
rs806366	rs806374	0.293	0.025
rs806366	rs806375	0.409	0.058
rs806366	rs806377	0.155	0.017
rs806366	rs806378	0.187	0.015
rs806366	rs2023239	0.139	0.014
rs806366	rs1535255	0.19	0.006
rs806366	rs6454672	0.208	0.007
rs1049353	rs806371	1	0.025
rs1049353	rs806374	0.642	0.019
rs1049353	rs806375	0.513	0.021
rs1049353	rs806377	0.314	0.012
rs1049353	rs806378	0.093	0.003
rs1049353	rs2023239	0.416	0.006
rs1049353	rs1535255	0.257	0.002
rs1049353	rs6454672	0.22	0.001
rs806371	**rs806374**	**0.922**	**0.459**

rs806371	rs806375	0.059	0.002
rs806371	rs806377	0.04	0
rs806371	rs806378	0.563	0.019
rs806371	rs2023239	0.171	0.02
rs806371	rs1535255	0.205	0.036
rs806371	rs6454672	0.161	0.024
rs806374	rs806375	0.114	0.008
rs806374	rs806377	0.283	0.032
rs806374	rs806378	0.467	0.026
rs806374	rs2023239	0.133	0.014
rs806374	rs1535255	0.25	0.039
rs806374	rs6454672	0.182	0.019
rs806375	rs806377	0.674	0.317
rs806375	rs806378	1	0.134
rs806375	rs2023239	0.8	0.294
rs806375	rs1535255	0.754	0.199
rs806375	rs6454672	0.817	0.213
rs806377	rs806378	0.151	0.002
rs806377	rs2023239	0.474	0.07
rs806377	rs1535255	1	0.245
rs806377	rs6454672	0.958	0.21
rs806378	rs2023239	0.159	0.008
rs806378	rs1535255	1	0.075
rs806378	rs6454672	1	0.068
rs2023239	**rs1535255**	**0.942**	**0.691**
rs2023239	**rs6454672**	**0.934**	**0.613**
rs1535255	**rs6454672**	**0.986**	**0.888**

Figure 5.4: Linkage Disequilibrium Plot computed with markers within the CNR1 gene in the GAP Black sample

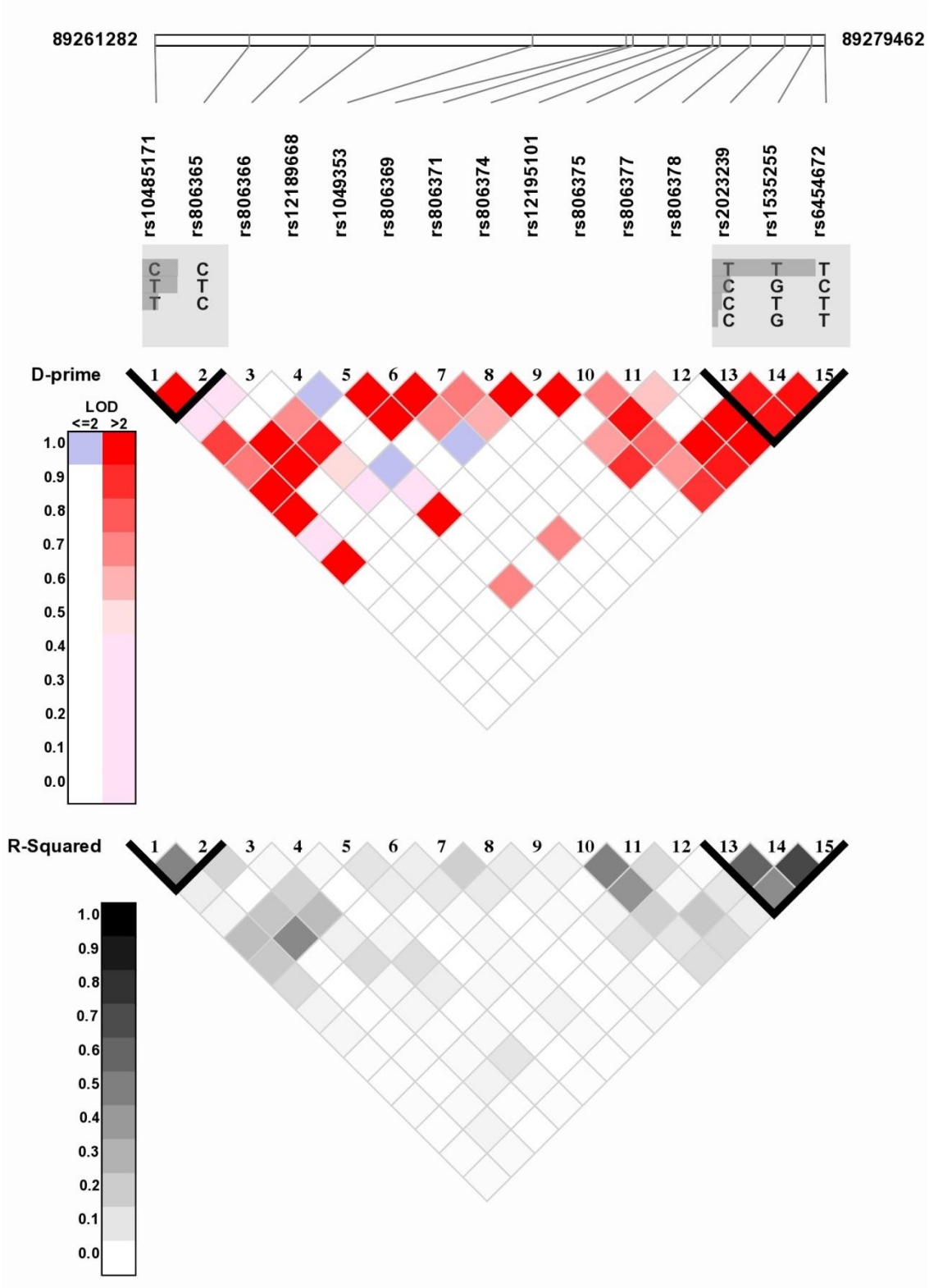


Table 5.10 D' and r2 values calculated with haploview in the GAP Black sample (shown in bold markers with higher D' and r2 scores further discussed in the conclusion paragraph of this chapter)

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs10485171	**rs806365**	**1**	**0.48**
rs10485171	rs806366	0.299	0.064
rs10485171	rs1049353	0.727	0.253
rs10485171	rs806371	1	0.14
rs10485171	rs806374	0.323	0.043
rs10485171	rs12195101	1	0.03
rs10485171	rs806375	0.114	0.009
rs10485171	rs806377	0.1	0.007
rs10485171	rs806378	0.206	0.012
rs10485171	rs2023239	0.143	0.004
rs10485171	rs1535255	0.194	0.011
rs10485171	rs6454672	0.235	0.012
rs806365	rs806366	0.49	0.157
rs806365	rs1049353	1	0.22
rs806365	rs806371	0.199	0.005
rs806365	rs806374	0.008	0
rs806365	rs12195101	0.311	0.005
rs806365	rs806375	0.099	0.006
rs806365	rs806377	0.107	0.008
rs806365	rs806378	0.046	0.001
rs806365	rs2023239	0.297	0.017
rs806365	rs1535255	0.547	0.043
rs806365	rs6454672	0.413	0.019
rs806366	rs1049353	0.686	0.169
rs806366	rs806371	0.505	0.054
rs806366	rs806374	0.472	0.138
rs806366	rs12195101	0.799	0.027
rs806366	rs806375	0.17	0.028
rs806366	rs806377	0.063	0.004
rs806366	rs806378	0.205	0.018
rs806366	rs2023239	0.391	0.045
rs806366	rs1535255	0.138	0.004
rs806366	rs6454672	0.081	0.001
rs1049353	rs806371	1	0.069
rs1049353	rs806374	0.134	0.004
rs1049353	rs12195101	0.003	0
rs1049353	rs806375	0.02	0
rs1049353	rs806377	0.258	0.022
rs1049353	rs806378	0.068	0.001
rs1049353	rs2023239	0.073	0.005

rs1049353	rs1535255	0.031	0
rs1049353	rs6454672	0.094	0.004
rs806371	rs806374	0.724	0.187
rs806371	rs12195101	0.609	0.079
rs806371	rs806375	0.011	0
rs806371	rs806377	0.133	0.003
rs806371	rs806378	0.035	0
rs806371	rs2023239	0.045	0.001
rs806371	rs1535255	0.382	0.006
rs806371	rs6454672	0.506	0.008
rs806374	rs12195101	1	0.073
rs806374	rs806375	0.213	0.026
rs806374	rs806377	0.161	0.015
rs806374	rs806378	0.074	0.001
rs806374	rs2023239	0.19	0.006
rs806374	rs1535255	0.377	0.017
rs806374	rs6454672	0.495	0.022
rs12195101	rs806375	1	0.042
rs12195101	rs806377	0.383	0.006
rs12195101	rs806378	0.642	0.041
rs12195101	rs2023239	0.894	0.112
rs12195101	rs1535255	0.828	0.006
rs12195101	rs6454672	0.407	0.001
rs806375	rs806377	0.711	0.493
rs806375	**rs806378**	**0.975**	**0.422**
rs806375	rs2023239	0.773	0.183
rs806375	rs1535255	0.66	0.098
rs806375	rs6454672	0.884	0.13
rs806377	rs806378	0.558	0.139
rs806377	rs2023239	0.316	0.031
rs806377	rs1535255	1	0.218
rs806377	rs6454672	0.945	0.144
rs806378	rs2023239	0.139	0.013
rs806378	rs1535255	1	0.091
rs806378	rs6454672	1	0.07
rs2023239	**rs1535255**	**0.948**	**0.605**
rs2023239	**rs6454672**	**0.964**	**0.448**
rs1535255	rs6454672	0.973	0.719

Figure 5.5: Linkage Disequilibrium Plot computed with markers within the CNR1 gene in the PICOS Study

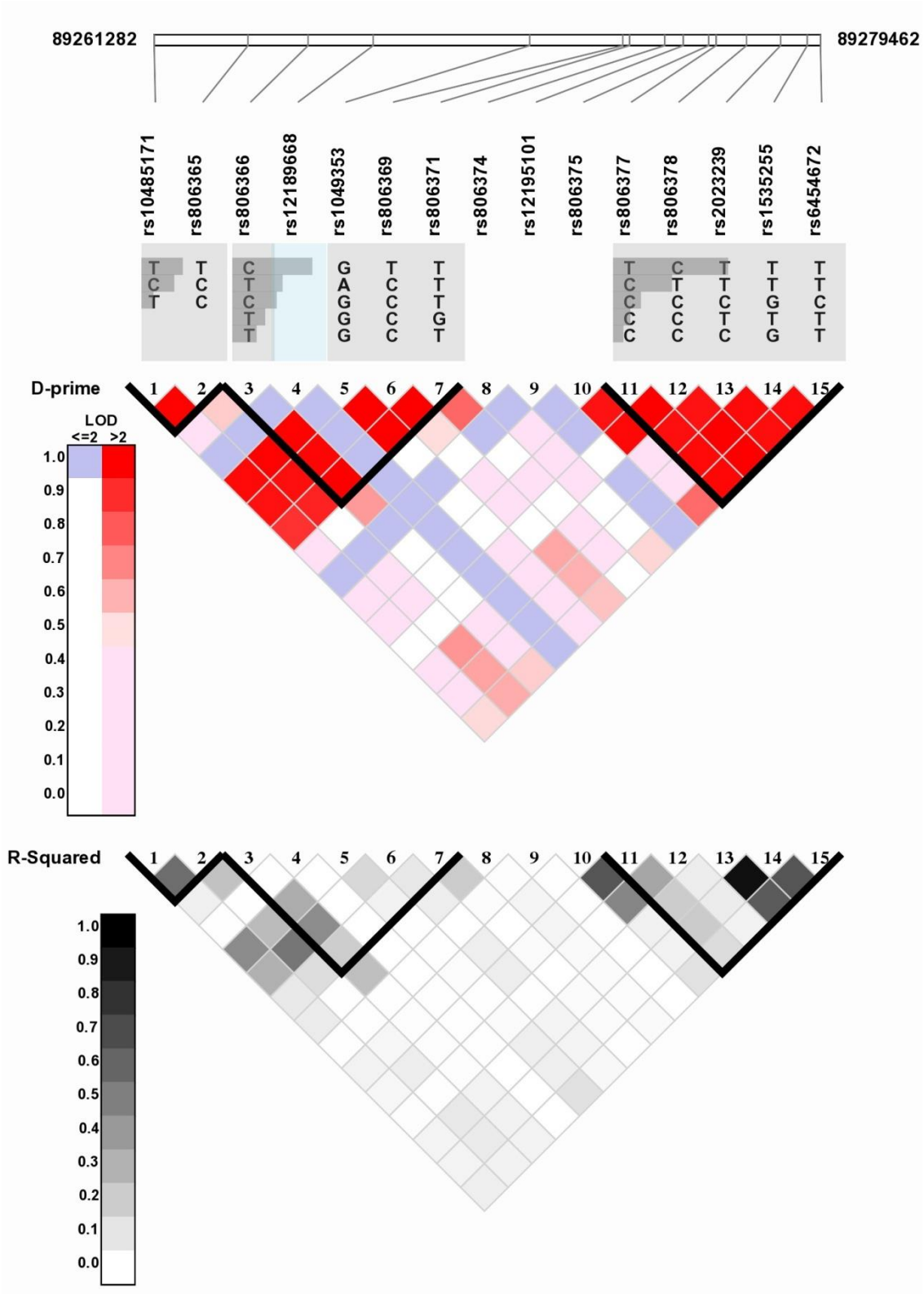


Table 5.11 D' and r2 values calculated with haploview in the PICOS Study sample (shown in bold markers with higher D' and r2 scores further discussed in the conclusion paragraph of this chapter)

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs10485171	**rs806365**	**0.992**	**0.566**
rs10485171	rs806366	0.279	0.055
rs10485171	rs1049353	0.978	0.45
rs10485171	rs806371	0.888	0.085
rs10485171	rs806374	0.49	0.074
rs10485171	rs12195101	1	0.001
rs10485171	rs806375	0.227	0.044
rs10485171	rs806377	0.221	0.03
rs10485171	rs806378	0.083	0.004
rs10485171	rs2023239	0.354	0.042
rs10485171	rs1535255	0.347	0.04
rs10485171	rs6454672	0.511	0.06
rs806365	rs806366	0.543	0.239
rs806365	rs1049353	0.992	0.265
rs806365	rs806371	0.922	0.13
rs806365	rs806374	0.041	0.001
rs806365	rs12195101	1	0.002
rs806365	rs806375	0.212	0.03
rs806365	rs806377	0.26	0.064
rs806365	rs806378	0.088	0.003
rs806365	rs2023239	0.664	0.086
rs806365	rs1535255	0.633	0.077
rs806365	rs6454672	0.613	0.05
rs806366	rs1049353	0.993	0.32
rs806366	rs806371	1	0.196
rs806366	rs806374	0.654	0.249
rs806366	rs12195101	1	0.002
rs806366	rs806375	0.086	0.006
rs806366	rs806377	0.091	0.007
rs806366	rs806378	0.149	0.007
rs806366	rs2023239	0.444	0.046
rs806366	rs1535255	0.453	0.049
rs806366	rs6454672	0.539	0.047
rs1049353	rs806371	1	0.048
rs1049353	rs806374	0.146	0.003
rs1049353	rs12195101	1	0.001
rs1049353	rs806375	0.138	0.008
rs1049353	rs806377	0.068	0.001
rs1049353	rs806378	0.347	0.012

rs1049353	rs2023239	0.289	0.06
rs1049353	rs1535255	0.295	0.062
rs1049353	rs6454672	0.485	0.116
rs806371	rs806374	0.768	0.199
rs806371	rs12195101	1	0.011
rs806371	rs806375	0.052	0
rs806371	rs806377	0.294	0.015
rs806371	rs806378	0.027	0
rs806371	rs2023239	0.129	0.014
rs806371	rs1535255	0.129	0.013
rs806371	rs6454672	0.212	0.001
rs806374	rs12195101	1	0.004
rs806374	rs806375	0.329	0.039
rs806374	rs806377	0.268	0.036
rs806374	rs806378	0.307	0.017
rs806374	rs2023239	0.33	0.012
rs806374	rs1535255	0.327	0.011
rs806374	rs6454672	0.518	0.019
rs12195101	rs806375	1	0.001
rs12195101	rs806377	1	0.002
rs12195101	rs806378	0.309	0.001
rs12195101	rs2023239	1	0
rs12195101	rs1535255	1	0
rs12195101	rs6454672	1	0
rs806375	**rs806377**	**0.959**	**0.661**
rs806375	**rs806378**	**0.98**	**0.474**
rs806375	rs2023239	0.421	0.051
rs806375	rs1535255	0.411	0.048
rs806375	rs6454672	0.762	0.113
rs806377	rs806378	1	0.346
rs806377	rs2023239	0.967	0.197
rs806377	rs1535255	0.978	0.201
rs806377	rs6454672	0.983	0.137
rs806378	rs2023239	0.959	0.067
rs806378	rs1535255	1	0.072
rs806378	rs6454672	1	0.05
rs2023239	**rs1535255**	**0.977**	**0.934**
rs2023239	**rs6454672**	**0.968**	**0.641**
rs1535255	rs6454672	0.974	0.66

Disequilibrium values, D' and r2, are measures of non random association of alleles at two or more loci. D' values, in a given population, assess the probability for historical recombination (Mueller

J C, 2004), and refer to the rate at which the two alleles tend to be co-inherited. D' value ranges from 0 to 1; when the value deviates from 1 there is evidence of historical recombination (Mueller J C, 2004). D' values tend to be higher when they are calculated in small sample seizes and/or in the presence of rare alleles (Mueller J C, 2004), they tend to suffer from ceiling effect. R2 values measure the degree of independence; it is a preferred measure of a polymorphism given the other. R2 is, in fact, widely used in association studies. If 2 given polymorphisms have a high r2 value, they can act as proxies of each other. It is therefore very important to calculate both D' and r2 value in order to better understand a given haplotype.

As it can been seen in the LD plot generated with the GAP Study sample data Caucasian group, in block 1 at the far right of the figure, the 3 SNPs namely rs2023239, rs1535255 and rs6454672 have both a high D' and r2 value. What the values suggest is that these 3 markers do not seem to be independent of each other. The high D' value points out the fact that the above mentioned markers tend to be co-inherited 90% of the times and because the r2 value is also high, they can act as proxies. The haplotype made of rs2023239, rs1535255 and rs6454672 is therefore, not very informative.

In the plot generated with the GAP Study sample Black group, there also is a visible block on the far right side of the picture. In it, rs2023239 rs1535255 rs6454672 have a D' value of 0.94 and 0.96 and r2 value of 0.4 and 0.6. Assuming that the D' value is higher due to the sample size, the high r2 scores may indicate that we are not in the presence of independent signals from these 3 markers. The same interpretation can be safely made for rs10485171 and rs806365 which compose another block in the population analysed.

In the PICOS Study sample we have a bigger pattern of LD. As it can be seen from the LD plot (Figure 5.5), there are 3 blocks in the population examined. In the first block rs10485171 rs806365 have a D' value of almost 1 and r2 score of 0.6, the same in the second block for rs2023239 rs1535255 rs6454672, and in the third a even higher dependence can be observed, where rs806375 rs806377 have both D' and r2 value of almost 1.

This suggests a pattern of co-inheritance and a high dependence between all the mentioned markers. We are in the presence of a small sample with rarer allele (as discusses earlier with the MAFs), it is therefore important to pay attention to both measures, D' and r2, even though, r2 is more informative in this case. Although we are in the presence of a small sample size compared, for example, to the recent GWAS studies done in psychosis, the number of case-control pairs is not as small as to influence very much the D' scores.
All haplotypes were tested for association with psychosis using a sliding window of 3.

In the GAP Caucasian sample, 9 haplotypes within 5 windows showed significant p-value (Table 5.12). When corrected with the Bonferroni multiple testing correction (α value/number of windows), in the GAP Study Caucasian sample, only 4 haplotypes maintained significance:

SNPS	HAPLOTYPE	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value/Corrected P-value
rs806366|rs12189668|rs1049353	TTA	0.04572	0.1437	9.194	1	0.002/0.026
rs806366|rs12189668|rs1049353	TTG	0.6822	0.4682	11.37	1	0.0007/0.009
rs12189668|rs1049353|rs806369	TAC	0.03752	0.1303	9.364	1	0.002/0.026
rs1049353|rs806369|rs806371	ACT	0.04295	0.1385	9.205	1	0.002/0.026

In the GAP Black sample, 6 haplotypes within 4 windows showed significant p-values (Table 5.13), but none of them retained significance after Bonferroni multiple testing correction.

In the PICOS group 6, haplotypes within 5 windows showed significant p-value (Table 5.14), however only 1 haplotype retained significance after Bonferroni multiple testing correction:

SNPS	HAPLOTYPE	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value/Corrected P-value
rs806375|rs806377|rs806378	ACC	0.1162	0.0729	8.681	1	0.003/0.039

All Haplotypes that retained a significant p-value after Bonferroni correction in the GAP Study sample Caucasian group, contain rs1049353 that incidentally have shown significance in the association analysis (Bonferroni corrected p-value=0.03). Although the GAP Study sample Caucasian group consists of a very low number of cases and controls and both the allelic and the haplotype associations found, have to be seen in light of a very statistically under powered study, I proceeded to perform a conditional test to check for an independent effect of rs1049353. Not surprisingly, the test showed an independent significant signal of rs1049353 from the remaining SNPs forming the other haplotypes (rs806366, rs806369, rs12189668 and rs806371) (df=2; p-value = 0.0289). These results have to be interpreted in light of the small sample size and the number of tests performed with the same markers within the same sample size. The issue is further discussed in the conclusions section.

The ACC haplotype also showed significance in the PICOS Study sample. It contains rs806378, the SNP that was found to be associated with psychosis, although it did not retain significance after multiple testing correction (corrected p-value=0.45). Conditional test showed the presence of an independent significant signal of rs806378 (df=2; p-value=0.0432).

Table 5.12 Haplotype association analysis of markers within the CNR1 gene in the GAP Caucasian sample

SNPS	HAPLOTYPE	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value/Corrected P-value
rs10485171|rs806365|rs806366	OMNIBUS	NA	NA	6.361	6	0.384
rs10485171|rs806365|rs806366	CTC	0.0162	0.03427	0.9702	1	0.3246
rs10485171|rs806365|rs806366	TTC	0.12	0.179	1.738	1	0.1873
rs10485171|rs806365|rs806366	CCC	0.07828	0.1263	1.651	1	0.1988
rs10485171|rs806365|rs806366	TCC	0.05896	0.04817	0.1232	1	0.7256
rs10485171|rs806365|rs806366	TTT	0.1769	0.1261	1.05	1	0.3055
rs10485171|rs806365|rs806366	CCT	0.3291	0.3368	0.0153	1	0.9014
rs10485171|rs806365|rs806366	TCT	0.2206	0.1493	1.756	1	0.1851
rs806365|rs806366|rs12189668	OMNIBUS	NA	NA	3.497	3	0.3211
rs806365|rs806366|rs12189668	TCT	0.1363	0.2089	2.288	1	0.1304
rs806365|rs806366|rs12189668	CCT	0.1319	0.1656	0.5256	1	0.4685
rs806365|rs806366|rs12189668	TTT	0.1757	0.1279	0.9031	1	0.3419
rs806365|rs806366|rs12189668	CTT	0.5561	0.4976	0.7606	1	0.3831
rs806366|rs12189668|rs1049353	OMNIBUS	NA	NA	18.88	3	0.0002893
rs806366|rs12189668|rs1049353	**TTA**	**0.04572**	**0.1437**	**9.194**	**1**	**0.002/0.026**
rs806366|rs12189668|rs1049353	**CCG**	**0.005951**	**0.0371**	**4.817**	**1**	**0.03/0.39**
rs806366|rs12189668|rs1049353	CTG	0.2661	0.3509	2.027	1	0.1546
rs806366|rs12189668|rs1049353	**TTG**	**0.6822**	**0.4682**	**11.37**	**1**	**0.0007/0.009**
rs12189668|rs1049353|rs806369	OMNIBUS	NA	NA	12.41	3	0.006
rs12189668|rs1049353|rs806369	CGT	0.009429	0.02792	1.515	1	0.2183
rs12189668|rs1049353|rs806369	TGT	0.09756	0.1319	0.7056	1	0.4009
rs12189668|rs1049353|rs806369	**TAC**	**0.03752**	**0.1303**	**9.364**	**1**	**0.002/0.026**
rs12189668|rs1049353|rs806369	**TGC**	**0.8555**	**0.7099**	**8.34**	**1**	**0.004/0.052**
rs1049353|rs806369|rs806371	OMNIBUS	NA	NA	11.77	3	0.008
rs1049353|rs806369|rs806371	GCG	0.2855	0.2251	1.046	1	0.3065
rs1049353|rs806369|rs806371	GTT	0.1111	0.1672	1.689	1	0.1938
rs1049353|rs806369|rs806371	**ACT**	**0.04295**	**0.1385**	**9.205**	**1**	**0.002/0.026**
rs1049353|rs806369|rs806371	GCT	0.5605	0.4691	1.917	1	0.1662
rs806369|rs806371|rs806374	OMNIBUS	NA	NA	2.904	5	0.7148
rs806369|rs806371|rs806374	CGC	0.2756	0.2321	0.5411	1	0.462
rs806369|rs806371|rs806374	TTC	0.01025	0.01849	0.3291	1	0.5662
rs806369|rs806371|rs806374	CTC	0.1416	0.1254	0.1226	1	0.7262
rs806369|rs806371|rs806374	CGT	0.01398	0.003143	0.5519	1	0.4575
rs806369|rs806371|rs806374	TTT	0.1027	0.158	1.719	1	0.1899
rs806369|rs806371|rs806374	CTT	0.4558	0.4628	0.011	1	0.9164
rs806371|rs806374|rs12195101	OMNIBUS	NA	NA	2.01	5	0.8478
rs806371|rs806374|rs12195101	GCC	0.02902	0.0396	0.2094	1	0.6472
rs806371|rs806374|rs12195101	TCC	0.01258	0.01922	0.1824	1	0.6693
rs806371|rs806374|rs12195101	GCT	0.2441	0.1923	0.8378	1	0.36
rs806371|rs806374|rs12195101	TCT	0.1369	0.1228	0.0952	1	0.7577
rs806371|rs806374|rs12195101	GTT	0.01414	0.003134	0.5629	1	0.4531
rs806371|rs806374|rs12195101	TTT	0.5633	0.623	0.8161	1	0.3663
rs806374|rs12195101|rs806375	OMNIBUS	NA	NA	2.175	4	0.7037
rs806374|rs12195101|rs806375	CTA	0.1865	0.153	0.4258	1	0.5141
rs806374|rs12195101|rs806375	TTA	0.2655	0.2735	0.0184	1	0.8922
rs806374|rs12195101|rs806375	CCT	0.04246	0.05882	0.3469	1	0.5559
rs806374|rs12195101|rs806375	CTT	0.1983	0.146	1.003	1	0.3166
rs806374|rs12195101|rs806375	TTT	0.3072	0.3687	0.9791	1	0.3224
rs12195101|rs806375|rs806377	OMNIBUS	NA	NA	1.947	5	0.8564
rs12195101|rs806375|rs806377	TAT	0.3012	0.2956	0.0084	1	0.9268
rs12195101|rs806375|rs806377	CTT	0.02122	0.04132	0.9571	1	0.3279
rs12195101|rs806375|rs806377	TTT	0.03044	0.05055	0.7049	1	0.4011
rs12195101|rs806375|rs806377	TAC	0.1512	0.1254	0.3064	1	0.5799

rs12195101|rs806375|rs806377	CTC	0.02048	0.01582	0.0646	1	0.7994
rs12195101|rs806375|rs806377	TTC	0.4754	0.4713	0.004	1	0.9498
rs806375|rs806377|rs806378	OMNIBUS	NA	NA	9.647	5	0.08589
rs806375|rs806377|rs806378	**TTT**	**0.04183**	**0.1048**	**4.502**	**1**	**0.03/0.39**
rs806375|rs806377|rs806378	**TCT**	**0.07788**	**0.1524**	**3.831**	**1**	**0.05/0.65**
rs806375|rs806377|rs806378	ATC	0.2962	0.2826	0.0511	1	0.8212
rs806375|rs806377|rs806378	TTC	0.01342	0.004024	0.4369	1	0.5086
rs806375|rs806377|rs806378	ACC	0.1555	0.1356	0.1763	1	0.6745
rs806375|rs806377|rs806378	TCC	0.4151	0.3206	2.137	1	0.1438
rs806377|rs806378|rs2023239	OMNIBUS	NA	NA	8.899	4	0.06367
rs806377|rs806378|rs2023239	**TTC**	**0.03654**	**0.09214**	**3.99**	**1**	**0.05/0.65**
rs806377|rs806378|rs2023239	CCC	0.3208	0.2507	1.32	1	0.2506
rs806377|rs806378|rs2023239	**CTT**	**0.08678**	**0.165**	**3.867**	**1**	**0.05/0.65**
rs806377|rs806378|rs2023239	TCT	0.315	0.3037	0.0342	1	0.8533
rs806377|rs806378|rs2023239	CCT	0.2409	0.1885	0.8869	1	0.3463
rs806378|rs2023239|rs1535255	OMNIBUS	NA	NA	8.418	4	0.0774
rs806378|rs2023239|rs1535255	CCG	0.3075	0.2314	1.58	1	0.2088
rs806378|rs2023239|rs1535255	TCT	0.03625	0.08765	3.441	1	0.0636
rs806378|rs2023239|rs1535255	CCT	0.01951	0.029	0.2454	1	0.6204
rs806378|rs2023239|rs1535255	TTT	0.08535	0.1623	3.751	1	0.05277
rs806378|rs2023239|rs1535255	CTT	0.5514	0.4896	0.8679	1	0.3515
rs2023239|rs1535255|rs6454672	OMNIBUS	NA	NA	4.548	4	0.3369
rs2023239|rs1535255|rs6454672	CGC	0.2857	0.2143	1.476	1	0.2244
rs2023239|rs1535255|rs6454672	TGC	0.01728	0.01429	0.0313	1	0.8595
rs2023239|rs1535255|rs6454672	CGT	0.02296	0.01429	0.2065	1	0.6495
rs2023239|rs1535255|rs6454672	CTT	0.05419	0.1143	3.415	1	0.0646
rs2023239|rs1535255|rs6454672	TTT	0.6199	0.6429	0.1292	1	0.7192

Analysis conducted using PLINK with a sliding window of three.

Significant p-values are shown in Bold.

*DF= Degrees of Freedom

Table 5.13 Haplotype association analysis of markers within the CNR1 gene in the GAP Study Black sample

SNPS	HAPLOTYPE	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value/Corrected P-value
rs10485171|rs806365|rs806366	OMNIBUS	NA	NA	6.406	5	0.2687
rs10485171|rs806365|rs806366	TTT	0.1095	0.08652	0.4915	1	0.4832
rs10485171|rs806365|rs806366	CCT	0.2935	0.246	0.9492	1	0.3299
rs10485171|rs806365|rs806366	TCT	0.1213	0.1135	0.04877	1	0.8252
rs10485171|rs806365|rs806366	TTC	0.3186	0.288	0.3671	1	0.5446
rs10485171|rs806365|rs806366	CCC	0.1094	0.1759	3.108	1	0.07789
rs10485171|rs806365|rs806366	TCC	0.04766	0.09013	2.456	1	0.1171
rs806365|rs806366|rs12189668	OMNIBUS	NA	NA	5.531	5	0.3546
rs806365|rs806366|rs12189668	TTC	0.0257	0.01723	0.2789	1	0.5974
rs806365|rs806366|rs12189668	CCC	0.0308	0.05248	1.029	1	0.3105
rs806365|rs806366|rs12189668	TTT	0.1086	0.07987	0.7945	1	0.3727
rs806365|rs806366|rs12189668	CTT	0.3902	0.3493	0.6001	1	0.4385
rs806365|rs806366|rs12189668	TCT	0.2937	0.2687	0.2573	1	0.612
rs806365|rs806366|rs12189668	CCT	0.1511	0.2323	3.674	1	0.05528
rs806366|rs12189668|rs1049353	OMNIBUS	NA	NA	4.415	5	0.4913
rs806366|rs12189668|rs1049353	TTA	0.248	0.1642	3.557	1	0.05931
rs806366|rs12189668|rs1049353	CTA	0.03608	0.03514	0.0022	1	0.9626
rs806366|rs12189668|rs1049353	TCG	0.01376	0.009027	0.1633	1	0.6861
rs806366|rs12189668|rs1049353	CCG	0.03754	0.05577	0.6515	1	0.4196
rs806366|rs12189668|rs1049353	TTG	0.2643	0.2716	0.02271	1	0.8802
rs806366|rs12189668|rs1049353	CTG	0.4003	0.4643	1.417	1	0.2338
rs12189668|rs1049353|rs806369	OMNIBUS	NA	NA	9.018	4	0.06066
rs12189668|rs1049353|rs806369	CGT	0.0219	0.01032	0.6834	1	0.4084
rs12189668|rs1049353|rs806369	TGT	0.2405	0.178	1.981	1	0.1593
rs12189668|rs1049353|rs806369	TAC	0.2869	0.2027	3.208	1	0.07326
rs12189668|rs1049353|rs806369	CGC	0.03352	0.05824	1.234	1	0.2666
rs12189668|rs1049353|rs806369	**TGC**	**0.4171**	**0.5508**	**6.095**	**1**	**0.013/0.13**
rs1049353|rs806369|rs806371	OMNIBUS	NA	NA	9.828	3	0.02009
rs1049353|rs806369|rs806371	GCG	0.1431	0.2044	2.273	1	0.1316
rs1049353|rs806369|rs806371	GTT	0.2664	0.1802	3.577	1	0.05857
rs1049353|rs806369|rs806371	ACT	0.2847	0.2017	3.142	1	0.07629
rs1049353|rs806369|rs806371	**GCT**	**0.3057**	**0.4137**	**4.358**	**1**	**0.04/0.52**
rs806369|rs806371|rs806374	OMNIBUS	NA	NA	4.494	5	0.4807
rs806369|rs806371|rs806374	CGC	0.1292	0.1616	0.7174	1	0.397
rs806369|rs806371|rs806374	TTC	0.03414	0.01576	1.112	1	0.2917
rs806369|rs806371|rs806374	CTC	0.2084	0.1943	0.1047	1	0.7462
rs806369|rs806371|rs806374	CGT	0.02011	0.03994	1.184	1	0.2765
rs806369|rs806371|rs806374	TTT	0.2288	0.1728	1.613	1	0.2041
rs806369|rs806371|rs806374	CTT	0.3793	0.4156	0.462	1	0.4967
rs806371|rs806374|rs12195101	OMNIBUS	NA	NA	3.322	5	0.6504
rs806371|rs806374|rs12195101	GCC	0.02469	0.03278	0.2051	1	0.6507
rs806371|rs806374|rs12195101	TCC	0.01031	0.01424	0.1116	1	0.7383
rs806371|rs806374|rs12195101	GCT	0.09924	0.1368	1.186	1	0.2762
rs806371|rs806374|rs12195101	TCT	0.2313	0.1934	0.729	1	0.3932
rs806371|rs806374|rs12195101	GTT	0.02094	0.04173	1.279	1	0.2581
rs806371|rs806374|rs12195101	TTT	0.6135	0.581	0.3767	1	0.5394
rs806374|rs12195101|rs806375	OMNIBUS	NA	NA	0.8157	4	0.9363
rs806374|rs12195101|rs806375	CCT	0.03571	0.04795	0.3188	1	0.5723
rs806374|rs12195101|rs806375	CTT	0.08773	0.1021	0.2018	1	0.6532
rs806374|rs12195101|rs806375	TTT	0.3562	0.3686	0.05598	1	0.813
rs806374|rs12195101|rs806375	CTA	0.2457	0.2199	0.3099	1	0.5777
rs806374|rs12195101|rs806375	TTA	0.2747	0.2615	0.07401	1	0.7856
rs12195101|rs806375|rs806377	OMNIBUS	NA	NA	5.158	4	0.2714
rs12195101|rs806375|rs806377	TTC	0.4109	0.4206	0.03234	1	0.8573
rs12195101|rs806375|rs806377	**TAC**	**0.05037**	**0.1069**	**3.894**	**1**	**0.05/0.065**

rs12195101|rs806375|rs806377	CTT	0.03009	0.03416	0.04509	1	0.8318
rs12195101|rs806375|rs806377	TTT	0.0359	0.04665	0.249	1	0.6178
rs12195101|rs806375|rs806377	TAT	0.4728	0.3917	2.23	1	0.1353
rs806375|rs806377|rs806378	OMNIBUS	NA	NA	7.833	4	0.09791
rs806375|rs806377|rs806378	TCT	0.2329	0.2032	0.43	1	0.512
rs806375|rs806377|rs806378	TTT	0.06962	0.08645	0.3337	1	0.5635
rs806375|rs806377|rs806378	TCC	0.1696	0.2227	1.516	1	0.2182
rs806375|rs806377|rs806378	**ACC**	**0.04763**	**0.1073**	**4.375**	**1**	**0.04/0.52**
rs806375|rs806377|rs806378	ATC	0.4803	0.3804	3.395	1	0.06541
rs806377|rs806378|rs2023239	OMNIBUS	NA	NA	6.828	4	0.1453
rs806377|rs806378|rs2023239	TTC	0.05752	0.07514	0.4373	1	0.5085
rs806377|rs806378|rs2023239	**CCC**	**0.1241**	**0.2053**	**4.232**	**1**	**0.04/0.52**
rs806377|rs806378|rs2023239	CTT	0.2502	0.2182	0.486	1	0.4857
rs806377|rs806378|rs2023239	CCT	0.08249	0.111	0.8129	1	0.3673
rs806377|rs806378|rs2023239	TCT	0.4857	0.3904	3.157	1	0.07562
rs806378|rs2023239|rs1535255	OMNIBUS	NA	NA	4.122	3	0.2486
rs806378|rs2023239|rs1535255	CCG	0.1411	0.2129	3.074	1	0.07956
rs806378|rs2023239|rs1535255	TCT	0.05625	0.07787	0.6492	1	0.4204
rs806378|rs2023239|rs1535255	TTT	0.2486	0.2188	0.4166	1	0.5186
rs806378|rs2023239|rs1535255	CTT	0.554	0.4904	1.372	1	0.2414
rs2023239|rs1535255|rs6454672	OMNIBUS	NA	NA	4.748	3	0.1912
rs2023239|rs1535255|rs6454672	CGC	0.1138	0.1487	0.9221	1	0.3369
rs2023239|rs1535255|rs6454672	CGT	0.02987	0.05727	1.599	1	0.2061
rs2023239|rs1535255|rs6454672	CTT	0.05561	0.08942	1.488	1	0.2226
rs2023239|rs1535255|rs6454672	**TTT**	**0.8007**	**0.7046**	**4.289**	**1**	**0.04/0.52**

Analysis conducted using PLINK with a sliding window of three.

Significant p-values are shown in Bold.

*DF= Degrees of Freedom

Table 5.14 Haplotype association analysis of markers within the CNR1 gene in the PICOS Study sample

SNPS	HAPLOTYPE	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value/Corrected P-value
rs10485171|rs806365|rs806366	OMNIBUS	NA	NA	8.049	4	0.08978
rs10485171|rs806365|rs806366	TTT	0.09929	0.1031	0.06023	1	0.8061
rs10485171|rs806365|rs806366	CCT	0.2354	0.2277	0.1274	1	0.7212
rs10485171|rs806365|rs806366	**TCT**	**0.1615**	**0.1179**	**6.207**	**1**	**0.012/0.156**
rs10485171|rs806365|rs806366	TTC	0.3704	0.3881	0.5055	1	0.4771
rs10485171|rs806365|rs806366	CCC	0.1334	0.1632	2.607	1	0.1064
rs806365|rs806366|rs12189668	OMNIBUS	NA	NA	4.499	3	0.2124
rs806365|rs806366|rs12189668	TTT	0.101	0.1025	0.009276	1	0.9233
rs806365|rs806366|rs12189668	**CTT**	**0.3939**	**0.3456**	**3.824**	**1**	**0.05/0.65**
rs806365|rs806366|rs12189668	TCT	0.3663	0.3875	0.7213	1	0.3957
rs806365|rs806366|rs12189668	CCT	0.1388	0.1644	1.904	1	0.1677
rs806366|rs12189668|rs1049353	OMNIBUS	NA	NA	4.348	2	0.1137
rs806366|rs12189668|rs1049353	TTA	0.2242	0.2211	0.02006	1	0.8874
rs806366|rs12189668|rs1049353	TTG	0.2708	0.2278	3.761	1	0.05246
rs806366|rs12189668|rs1049353	CTG	0.505	0.551	3.194	1	0.07393
rs12189668|rs1049353|rs806369	OMNIBUS	NA	NA	0.004099	2	0.998
rs12189668|rs1049353|rs806369	TGT	0.3461	0.3464	0.0001647	1	0.9898
rs12189668|rs1049353|rs806369	TAC	0.224	0.2227	0.003939	1	0.95
rs12189668|rs1049353|rs806369	TGC	0.4299	0.4309	0.001636	1	0.9677
rs1049353|rs806369|rs806371	OMNIBUS	NA	NA	4.165	3	0.2442
rs1049353|rs806369|rs806371	GCG	0.1631	0.1295	3.505	1	0.06117
rs1049353|rs806369|rs806371	GTT	0.348	0.3473	0.0007561	1	0.9781
rs1049353|rs806369|rs806371	ACT	0.215	0.2199	0.05385	1	0.8165
rs1049353|rs806369|rs806371	GCT	0.274	0.3032	1.575	1	0.2094
rs806369|rs806371|rs806374	OMNIBUS	NA	NA	4.716	5	0.4515
rs806369|rs806371|rs806374	CGC	0.1427	0.1131	3.066	1	0.07992
rs806369|rs806371|rs806374	TTC	0.05017	0.06104	0.8397	1	0.3595
rs806369|rs806371|rs806374	CTC	0.148	0.1559	0.181	1	0.6705
rs806369|rs806371|rs806374	CGT	0.02495	0.02069	0.3179	1	0.5729
rs806369|rs806371|rs806374	TTT	0.2948	0.2852	0.1696	1	0.6804
rs806369|rs806371|rs806374	CTT	0.3394	0.3641	1.012	1	0.3143
rs806371|rs806374|rs12195101	OMNIBUS	NA	NA	4.038	3	0.2574
rs806371|rs806374|rs12195101	GCT	0.141	0.1108	3.215	1	0.07297
rs806371|rs806374|rs12195101	TCT	0.199	0.2182	0.8378	1	0.36
rs806371|rs806374|rs12195101	GTT	0.02564	0.02072	0.4153	1	0.5193
rs806371|rs806374|rs12195101	TTT	0.6345	0.6503	0.4157	1	0.5191
rs806374|rs12195101|rs806375	OMNIBUS	NA	NA	2.836	3	0.4176
rs806374|rs12195101|rs806375	CTT	0.08864	0.09521	0.1931	1	0.6604
rs806374|rs12195101|rs806375	TTT	0.302	0.3378	2.208	1	0.1373
rs806374|rs12195101|rs806375	CTA	0.2492	0.2332	0.5272	1	0.4678
rs806374|rs12195101|rs806375	TTA	0.3602	0.3337	1.17	1	0.2793
rs12195101|rs806375|rs806377	OMNIBUS	NA	NA	8.965	2	0.01131
rs12195101|rs806375|rs806377	TTC	0.3825	0.4303	3.528	1	0.06033
rs12195101|rs806375|rs806377	**TAC**	**0.1162**	**0.07547**	**7.536**	**1**	**0.006/0.07**
rs12195101|rs806375|rs806377	TAT	0.5013	0.4942	0.07536	1	0.7837
rs806375|rs806377|rs806378	OMNIBUS	NA	NA	12.93	3	0.0048
rs806375|rs806377|rs806378	**TCT**	**0.2203**	**0.278**	**6.517**	**1**	**0.01/0.13**
rs806375|rs806377|rs806378	TCC	0.1599	0.1523	0.1641	1	0.6854
rs806375|rs806377|rs806378	**ACC**	**0.1162**	**0.0729**	**8.681**	**1**	**0.003/0.039**
rs806375|rs806377|rs806378	ATC	0.5035	0.4968	0.06782	1	0.7945
rs806377|rs806378|rs2023239	OMNIBUS	NA	NA	8.026	3	0.04547
rs806377|rs806378|rs2023239	CCC	0.1857	0.1562	2.373	1	0.1234
rs806377|rs806378|rs2023239	**CTT**	**0.2194**	**0.2785**	**6.929**	**1**	**0.008/0.1**
rs806377|rs806378|rs2023239	CCT	0.08348	0.07204	0.7034	1	0.4016
rs806377|rs806378|rs2023239	TCT	0.5114	0.4933	0.4961	1	0.4812

rs806378|rs2023239|rs1535255	OMNIBUS	NA	NA	6.875	2	0.03214
rs806378|rs2023239|rs1535255	CCG	0.1854	0.1612	1.556	1	0.2123
rs806378|rs2023239|rs1535255	**TTT**	**0.2232**	**0.281**	**6.535**	**1**	**0.01/0.13**
rs806378|rs2023239|rs1535255	CTT	0.5914	0.5578	1.735	1	0.1878
rs2023239|rs1535255|rs6454672	OMNIBUS	NA	NA	2.328	2	0.3123
rs2023239|rs1535255|rs6454672	CGC	0.1396	0.115	2.098	1	0.1475
rs2023239|rs1535255|rs6454672	CGT	0.04895	0.04507	0.1276	1	0.7209
rs2023239|rs1535255|rs6454672	TTT	0.8114	0.8399	2.159	1	0.1417

Analysis conducted using PLINK with a sliding window of three.

Significant p-values are shown in Bold.

*DF= Degrees of Freedom

5.7 Summary of results

The summarized results of statistical analyses performed in this chapter are as follow:

- 15 tag SNPs within the CNR1 gene were analysed for main effect on psychosis in 3 different study samples (GAP Caucasian; GAP Black and PICOS)
- A χ^2 test was performed to check for allelic as well as genotypic association of each SNPs and psychosis. rs1049353 showed positive association in the GAP Caucasian sample, also after conservative Bonferroni correction (p-value=0.03). rs806378 showed marginal positive association in the GAP Caucasian sample and in the PICOS sample, it only retained significance after multiple testing correction in the GAP Caucasian sample (p-value=0.05). Finally, rs806371 showed positive association in the PICOS sample but not after Bonferroni correction.
- After haplotype analysis with window of 3, 4 haplotypes (rs806366-rs12189668-rs1049353) TTA; (rs806366-rs12189668rs1049353) TTG; (rs12189668-rs1049353-rs806369) TAC; (rs1049353-rs806369-rs806371) ACT showed positive association also after multiple testing correction (p-value=0.03, 0.009, 0.03 and 0.03 respectively; target Bonferroni p-value=0.005). In the PICOS group, 1 haplotype showed positive association (rs806375-rs806377-rs806378) ACC after Bonferroni multiple testing correction (p-value=0.003).
- The 4 haplotypes found significant in the GAP Caucasian sample all contained rs1049353. I therefore performed a conditional test to check for independent signal from the SNP. Results showed that indeed there was significance (p-value=0.02).

- The ACC haplotype found significant in the PICOS Study sample contained rs806378. I therefore performed a conditional test to check for independent signal from the SNP. Results showed significance (p-value=0.04).
- No association was found between rs1049353 and cannabis use. The SNP did not seem to have a moderating effect on cannabis use.
- No gene x environment interaction was observed between rs1049353 and cannabis use.

5.8 Conclusions

It is of a great importance to interpret the results shown on the basis of many limitations of this study. First and foremost, one of the biggest obstacles resides in the sample size with the GAP Study sample Caucasian group consisting of 174 psychotic patients and 45 controls. This sample size seems to be more relevant because of the association found for rs1049353 with psychosis, a similar effect also seen in the haplotype analysis. Small sample size, does not only limit statistical power of discovery, which is very low in this study. A small sample size, may also give a false estimation of MAFs, this is to say that some alleles could be under represented, also because of the number of subjects genotyped. For example, rs1049353 is a common polymorphisms of which the minor A allele frequency is 0.23 in the CEU population of HapMap. In this study the MAF of affected Vs unaffected individuals is 0.04/0.14 respectively. Such difference may indeed reflect disease status and normal genetic frequency variation across different Caucasian populations, but could also be due to the very low number of subjects genotyped. This, in turn, may generate false positives with p-values retaining significance even after the strictest multiple testing correction. Low MAFs and sample size also influence the values of D' calculated in a given population, therefore it is important to also pay attention to the r2 values calculated in order to check for the presence of proxy SNPs. In the GAP Caucasian group rs1049353 do not seem to however; be closely related to any of the neighbouring SNPs as can be seen in the table below. In particular, rs1049353-rs806371 show a D' value of 1 and a r2 score of 0.025. This is very likely due to the ceiling effect of D' value due, as mentioned earlier, to a very small sample size. Therefore, although D' is high, r2 is very low and it can be concluded that these 2 SNPs are not in strong LD. The same consideration can be made for SNPs rs1049353-rs806374 and rs1049353-rs806375 and others that can be fully seen in table 5.7. This could give the confidence that the signal, if not a statistical false positive, is derived solely from rs1049353.

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs1049353	rs806371	1	0.025
rs1049353	rs806374	0.642	0.019
rs1049353	rs806375	0.513	0.021
rs1049353	rs806377	0.314	0.012

The positive association shown in the haplotype analysis in the GAP Study sample Caucasian group and the PICOS Study sample can also be interpreted in the same way as allelic association. It is in fact very important to note that the haplotype frequency is low, being in the psychotic and non psychotic population within the GAP Caucasian group of 0.4 and 0.14 respectively (CTA haplotype) and 0.05 and 0.14 (TAT haplotype). The markers were not in high LD with r2 score below 0.2, but the low frequency together with a very small sample size, have probably generated a statistical false positive result. The same considerations can be made for the haplotype that survived the Bonferroni correction in the PICOS Study sample: rs806375|rs806377|rs806378 (ACC). The haplotype frequency in psychotic patients and non psychotic controls was low (0.12 and 0.07 respectively) and the markers scored in the high range for D' and r2 values:

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs806375	rs806377	0.959	0.661
rs806375	rs806378	0.98	0.474
rs806377	rs806378	1	0.346

Bonferroni multiple testing correction is very strict, it is common place to use other type of tests like permutation tests. Surviving Bonferroni correction though, does not equal true association; it is still possible to be in the presence of a false positive. Indeed, many SNPs in the PICOS Study sample do not show a completely independent signal from each others, although they are tag SNPs. This is, in turn, also partly explained by low haplotype frequency and small sample size.

In the literature, there are conflicting reports of these SNPs being associated with metabolic disorder, schizophrenia, anxiety and drug abuse. Among all markers found in literature, rs1049353 seem to be the most studied. It is a A/G base change located in the coding region of the Cannabinoid Receptor 1 gene (Exon 4) with both alleles described as risk alleles by different studies (Hadmani et al., 2008) (De Luis et al., 2012). Rs1049353 has been found to be associated with cocaine dependence (Zuo et al., 2009), major depression (Mitjans et al., 2013), lack of improvement in leptin levels (De Luis et al., 2012) and very recently with schizophrenia, although it did not pass the multiple correction (Costa et al., 2013). Rs1049353 has been reported associated with cigarette intake (Bienertova-Vaskus et al., 2012), but no evidence of association was found with other main

stream drugs of abuse (Benyamina et al., 2011). Incidentally, rs1049353 is the SNP that returned lower p-values in this thesis. It showed a main effect on psychosis in the GAP Caucasian population but no evidence of association with cannabis use or gene x environment interaction.

In light of the limitations highlighted earlier, but also in light of many evidences presented in the literature; results of this work are not seen as a striking positive association. However, neither should they be treated as certain false positive. They are interpreted as indication of a possible involvement of the CNR1 gene in the risk for psychosis. Further work needs to be done if the role of rs1049353 and more in general on the CNR1 gene needs to be elucidated in the pathophysiology of schizophrenia. The first suggestion would be increasing the sample size to many more case-control matched pairs. This is in order to obtain a power of at least 80%, diminishing therefore the possibility of statistical error. As shown in the power calculation (Materials and Methods chapter) in order to detect an effect size of 1.2 in a model assuming a population prevalence of 1% and allelic log additive effect for a MAF of 0.1 and, around 4,000 cases and matched controls are needed. A smaller number of samples is needed if the effect size is bigger, but it is plausible that schizophrenia is a polygenic disorder with multiple genes of small size all playing a role in the pathophysiology. Another consideration would be on the genotyping methods used; in light of recent sequencing techniques being easier to perform and cheaper, it is only logical to move towards that new field with whole exome sequence of pairs of cases and controls ranging in the thousands. Increasing the sample size would limit the possibility of type 1 error and maximize the likelihood of finding a true association. Most recent GWAS have failed to report any association of the rs1049353 polymorphism in schizophrenia. One of the latest GWAS analysis performed is the one by Ripke et al., who analysed more than 11,000 population-based samples from Sweden and re-analysed data using 1000 genome imputation in over 20,000 samples, excluding the Swedish ones. No markers within the CNR1 or the COMT gene were found to be significantly associated with schizophrenia (Ripke et al., 2013). This suggests that we are in the presence of a false positive association that will disappear once full statistical power is achieved.

CHAPTER 6

RESULTS

The Catechol-O-Methyltransferase gene in First Episode of Psychosis

6.1 Background information

Catechol-O-Methyltransferase (COMT) catalyses the O-methylation of catecholamines including dopamine, adrenaline and noradrenalin. It was discovered by Axelrod and Tomchick in 1958 (Axelrod and Tomchick, 1958). COMT is expressed throughout the brain but it plays a particularly important role in the prefrontal cortex for dopamine flux, where inactivation of dopamine is preferentially performed by catabolic enzymes. The COMT gene is located on the short arm of chromosome 22: 22q11.2 and encodes two main isoforms: S-COMT, soluble and mostly found in human peripheral tissues (Jeffery and Roth, 1984) and MB-COMT, a longer membrane found mainly in the brain (Tenhunen et al., 1994). MB-COMT has much higher affinity for dopamine and noradrenaline than S-COMT.

Over the years, many polymorphisms within the COMT gene have been reported associated with schizophrenia; however, the evidence is still inconsistent. Meta-analysis studies in 2003 and 2005 reported no or very minimal association of COMT rs4680 polymorphism with schizophrenia in a case-control and family based cohort (Glatt et al., 2003) and Asian and European case-control population (Fan et al., 2005). A further meta-analysis investigating 4 more markers within the COMT gene (rs737865, rs165599, rs2075507, rs165849) also found no evidence of association with schizophrenia in a Japanese case-control population (Okochi et al., 2009). Interestingly though, Costas et al., found significant association between rs4680 and schizophrenia in a meta-analysis of 51 studies, under an over dominant model; where having too low as well as too high levels of Dopamine signaling seemed to be a risk factor for the disease (Costas et al., 2011). More recently, rs4680 has been associated with an increase in violent behavior among schizophrenic patients (Bhakta et al., 2012) and among schizophrenic male patients in a meta-analysis (Singh et al., 2012). An effect of rs4680 on schizophrenia was also described by Lo Bianco, who found evidence of a main effect in the striatum and an interaction between the Val/Val genotype and brain activity in prefrontal cortex during emotion processing (Lo Bianco et al., 2012). rs4680 was also associated with schizophrenia, together with rs4646315 and rs9332377, in a North Indian case-control study (Kukshal et al., 2013). Other studies, however failed to replicate any association (Lajin et al., 2011) (Zhang et al., 2012) (Chen et al., 2012) (Tovilla-Zarate et a., 2013). A number of studies have also implicated rs4680 in neuropsychological performances and brain structure, with evidences in temporal regions, frontal areas, lateral ventricles and thalamus (reviewed in Ira

et al., 2013). This review suggested a link between rs4680 and 2 neuropsychological and 2 brain structural endophenotypes.

Certainly, rs4680 is the most studied polymorphism within the COMT gene to date and, although conflicting, evidence suggests a possible role in the pathophysiology of schizophrenia. Rs4680 is a common polymorphism located in the coding region of the COMT gene, resulting in a valine/methionine substitution at codon 158 in the MB-COMT isoform (Met158Val). This substitution affects the thermostability of the enzyme resulting in reduction of COMT activity in the Met allele carriers and an increase of function in Val allele carriers (Lachman et al., 1996) with 38% reduction of COMT activity in PFC tissue in human post mortem studies, with no change in mRNA transcription (Chen et al., 2004). This leads to reduced degradation and therefore higher levels of catecholamines in the Met allele carriers. As reviewed in chapter 3 of this thesis, rs4680 has been implicated widely as having a possible role in the interaction with environment, particularly drug abuse. The first study to highlight the possible interaction between rs4680 and cannabis use was that of Caspi in 2005, who found that Val carriers had a five fold increased risk in developing schizophreniform disorder (Caspi et al., 2005). These findings were supported by the work of Henquet et al, in 2006, Estrada et al., 2011 and by mice models (O'Tualthaigh et al., 2012). Some conflicting evidence however, has come from the recent studies of Okochi and Zammit (Okochi et al., 2011) (Zammit et al., 2011). Furthermore, Sanders et al., found no evidence of association between the rs4680 polymorphism and schizophrenia in a large sample of over 3.500 subjects (Sanders et al., 2008).

In light of the reviewed evidence, rs4680 seemed to be one of the logical candidates to be analyzed in this chapter. Rs4680 is also part of a haplotype formed with 3 other polymorphisms, namely rs6269, rs4633 and rs4818. This haplotype seems to be a better index of COMT enzymatic activity than the single polymorphism. Nackley et al. analyzed the three different haplotypes made by rs6269, rs4633 and rs4818 within the COMT gene; they were named after their ability to influence sensitivity to pain: Low Pain Sensitivity (LPS) haplotype, Average Pain Sensitivity (APS) haplotype and High Pain Sensitivity (HPS) haplotype. The three haplotypes contain two synonymous and 1 non synonymous variations and both the HPS and the LPS contain the Val allele whereas the APS contained the Met allele at position 158 (Nackley et al., 2006). Both haplotypes containing the Val allele showed higher enzymatic activity (Nackley et al., 2006).

Rs165599 is located in the COMT 3'- untraslated region (UTR) and it is a G to A change. It has been found to be positively associated with Schizophrenia (Shifman et al., 2002) (Allen et al., 2008). Furthermore, rs165599 has been found to be preferentially transmitted to affected individuals and to account for a later onset of the disease in a family study of Tawanese schizophrenic population (Chien et al., 2009). The same study found no association with rs4680 and rs737865 and schizophrenia (Chien et al., 2009), with rs737865 being located in intron 1 of the gene. Jugurnauth and colleagues found rs165599 as part of a haplotype with rs4680 to be over represented in

Methamphetamine users but not in schizophrenia patients (Jugurnauth et al., 2011). Indeed Maria et al., showed a higher frequency of a haplotype made of rs4680, rs737865 and rs165599 in Greek schizophrenic patients (Maria et al., 2012). The same polymorphism was also found to be positively associated in a cohort of African patients (Wright et al., 2012). Rs2075507 is located in the promoter region 2 of the COMT gene and has been investigated in schizophrenia by several studies.

There is however no supporting evidence of a role in the increase of the risk of the disease (Nunokawa et al., 2007) (Okochi et al., 2009), although it showed significant association as part of the rs4680-rs165599-rs2075507 haplotype in a group of female group of schizophrenic patients with sensory gating deficits (Ancin et al., 2011).

The interaction between the COMT gene, particularly rs4680 and cannabis use, has been explored widely during the last decade. Since the publication of Caspi et al., in 2005 showed that the Val allele moderated the effect of cannabis in a schizophrenic population with a 5 fold increase of risk of the disease, replications have showed a mix of positive and negative findings (reviewed in chapter 3). As mentioned earlier, the LPS haplotype can represent better the enzymatic activity of the COMT gene and therefore may show a stronger interaction with cannabis use. Furthermore, consumption before or after adolescence seems to play an important role in the pathophysiology of the disorder, with earlier start of use posing a greater risk (Arsenault et al., 2002) (Stefanis et al., 2004) (Dragt et al., 2010) (Estrada et al., 2011).

6.2 Hypothesis under investigation

This chapter aims to investigate whether 7 SNPs within the COMT gene namely rs737865, rs6269, rs4633, rs4818, rs165599, rs4680 and rs2075507 have a main effect on psychosis.

I hypothesise that the analysed SNPs and the LPS haplotype within the COMT gene have a main effect on psychosis.

The LPS haplotype (a priori hypothesis) is also investigated in search for evidence of interaction with cannabis use. I hypothesise that the LPS haplotype moderates the risk of psychosis following use of cannabis.

Furthermore I will conduct exploratory analyses to examine the role of the LPS haplotype in moderating the risk of psychosis following high VS low frequent use of cannabis and use in adolescence.

Table 6.1 shows all markers analysed in this chapter with their chromosomal position, minor allele frequency as reported in NCBI database and base pairs sequence.

GENE	SNP	Chr. POSITION	MAF	SEQUENCE
COMT	rs6269	3801154	G=0.37	GCATTTCTGAACCTTGCCCCTCTGC[G/A]AACACAAGGGGGCGATGGTGGCACT
COMT	rs4633	3801437	T=0.39	CCAAGGAGCAGCGCATCCTGAACCA[C/T]GTGCTGCAGCATGCGGAGCCCGGGA
COMT	rs4818	3802409	G=0.32	GCCTGCTGTCACCAGGGGCGAGGCT[C/G]ATCACCATCGAGATCAACCCCGACT
COMT	rs4680	3802473	A=0.39	CCAGCGGATGGTGGATTTCGCTGGC[A/G]TGAAGGACAAGGTGTGCATGCCTGA
COMT	rs2075507	3779368	G=0.35	CTGGACTGTGAGTATGGGAAGGGGAA[A/G]CTTTTCTGTCTGTTGTCCCCACTAC
COMT	rs165599	3807982	G=0.46	TGTTAGCCCCATGGGGACGACTGCC[A/G]GCCTGGGAAACGAAGAGGAGTCAGC
COMT	rs737865	3781397	G=0.23	GCTTTTTGGATTTTTCCAGCCAGGG[A/G]TTTTTGTGTCCTGTTGCTTTTTATT

Table 6.1 List of markers selected within the COMT gene

6.3 SNPs Genotyping

Primers used to genotype were ordered through Applied Biosystems as follow:

rs6269 (Chr. 22 - 18329952) - Context-Sequence VIC/FAM
GCATTTCTGAACCTTGCCCCTCTGC[G/A]AACACAAGGGGGCGATGGTGGCACT

rs4633 (Chr. 22 - 18330235) - Context-Sequence VIC/FAM
CCAAGGAGCAGCGCATCCTGAACCA[C/T]GTGCTGCAGCATGCGGAGCCCGGGA

rs4818 (Chr. 22 – 18331207) - Context-Sequence VIC/FAM
GCCTGCTGTCACCAGGGGCGAGGCT[C/G]ATCACCATCGAGATCAACCCCGACT

rs4680 (Chr. 22 – 18331271) - Context-Sequence VIC/FAM
CCAGCGGATGGTGGATTTCGCTGGC[A/G]TGAAGGACAAGGTGTGCATGCCTGA

rs737865 (Chr. 22 – 19930121) - Context-Sequence VIC/FAM
GCTTTTTGGATTTTTCCAGCCAGGG[A/G]TTTTTGTGTCCTGTTGCTTTTTATT

rs165599 (Chr. 22 – 19956781) - Context-Sequence VIC/FAM
TGTTAGCCCCATGGGGACGACTGCC[A/G]GCCTGGGAAACGAAGAGGAGTCAGC

rs2075507 (Chr. 22) (Information obtained from Okochi and colleagues as reported in Okochi et al., 2009. The same set of Applied Biosystems Custom Assays was used)

rs2075507 (Assay-by-Design)	
RS2075507-SNP1F	CGTGTCTGGACTGTGAGTATGG
RS2075507-SNP1R	GGGTTCAGAATCACGGATGTGA
RS2075507-SNP1V2 (VIC)	ACAGAAAAGTTTCCCC
RS2075507-SNP1M2 (FAM)	CAGAAAAGCTTCCCC

Primers are at 900 nM final concentration.

PCR followed standard procedure as per Applied Biosystems TaqMan® SNP Genotyping Assays standard dry DNA protocol:

Reaction Component	**FINAL CONCENTRATION**	**VOLUME (µl)**
TaqMan Gene expression assay 20X Assay Mix	1X	0.1 µl
cDNA Template	10ηg	2. µl
ddH2O		0.9 µl
TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2X)	1X	1 µl

PCR reaction was carried out in the Applied Biosystems 7900HT Fast Real-Time PCR System machine. as per manufacturer protocol with the following conditions:
STEP 1 - hold at 50C for 2 minutes
STEP 2 - denature at 92C for 15 seconds
STEP 3 - anneal/extend at 60C for 1 minute
STEP 4 - repeat step 2 and 3 for 40 cycles.
Plates were then subjected to reading using the Applied Biosystems 7900HT Fast Real-Time PCR System.

6.4 Statistical Analysis

In this chapter samples analysed are from the GAP Study (both Caucasian and Black population included) and from the PICOS study.

The GAP sample was genotyped for 7 SNPs within the COMT gene: rs737865, rs6269, rs4633, rs4818, rs165599, rs4680 and rs2075507.

The PICOS samples were only genotyped for rs4680 within the COMT gene.

The GAP Study Caucasian population analysed consisted of 174 psychotic patients and 45 non psychotic subjects;

The GAP Study Black population analysed consisted of 113 psychotic patients and 95 non psychotic subjects;

The PICOS Study sample consisted of 347 psychotic patients and 307 non psychotic patients.

The three main groups of samples were tested separately: Caucasian group (GAP); Black group (GAP); Caucasian Italian group (PICOS).

Gene-environment interaction was only calculated for the GAP Caucasian and the GAP Black study samples because of lack of environmental data (Cannabis use) for the PICOS study sample. They were analysed as 2 separate samples.

The GAP Study Caucasian population analysed consisted of 174 psychotic patients (68 non cannabis users, 106 cannabis users) and 45 non psychotic subjects (20 non cannabis users, 25 cannabis users).

The GAP Study Black population analysed consisted of 113 psychotic patients (39 non cannabis users, 63 cannabis users, 11 excluded with no information) and 95 non psychotic subjects (29 non cannabis users, 56 cannabis users, 10 excluded with no information).

6.4.1 Statistical tests performed

With the three sets of samples, namely the GAP study sample (including Caucasian subjects), the GAP study (including the black population) and the PICOS study sample (consisting of Caucasian population) the following statistical tests were performed:

- Hardy Weinberg Equilibrium Test
- Logistic regression test for allelic and genotype association analysis between each SNP and disease status
- Logistic regression test for haplotype association analysis (only the GAP Caucasian and Black study sample)
- Logistic regression test for the Gene x Environment analysis (only the GAP Caucasian and Black study sample)
- Bonferroni multiple testing correction

6.4.1.1 Hardy Weinberg Equilibrium Test

Hardy Weinberg Equilibrium Test was performed using PLINK software for genetic analysis throughout the thesis (Purcell et al., 2007).

The command used for the HWE test is --hardy

The full line of command used to run the HWE test in this thesis is:

Plink --file mydata --hardy

A file containing three entries for each SNP is then generated and saved as plink.hwe

The file contains calculations of the HWE test for all, affected only or unaffected only subjects.

In this thesis, only results of the unaffected group of subjects is discussed are reported in the tables.

6.4.1.2 Allelic and genotype association analysis

Statistical analysis carried out to establish association between allelic and genotype variation at each locus within the COMT and psychosis was performed using PLINK software for genetic analysis (Purcell et al., 2007).

The main effect of each SNP on psychosis was tested with logistic regression. PLINK calculates a basic association of a disease trait by comparing allele frequencies between cases and controls (Purcell et al., 2007). The association commands used are --assoc --logistic.

SNPs alleles and genotypes were treated as independent variables and disease outcome (psychosis) was treated as dependent variable. It was coded in the program as a binary disease status: 1=case 0=control. Because the disease outcome was a binary value, --1 was added to the command line used.

The complete set of commands used to calculate association in this thesis was:

Plink –file mydata --1 --assoc

A file containing both allelic and genotype association calculation is then created and saved as plink.assoc.

6.4.1.3 Haplotype analysis

Linkage Disequilibrium between SNPs within the COMT gene was computed using Haploview version 3.32 (Barret et al., 2005).

A graphical representation of linkage disequilibrium between the 4 markers constituting the LPS haplotype was created with LD-Plus, a program that is freely available via a web interface (https://chgr.mc.vanderbilt.edu/ldplus). D’ and r2 values, were calculated using Haploview (Barrett et al., 2005).

A graphical representation of linkage disequilibrium between all 7 analysed markers within the COMT was created with haploview (Barrett et al., 2005).

Haplotype analysis was then carried out using PLINK software for genetic analysis (Purcell et al., 2007).

In order to phase a set of SNPs for haplotypic analysis, both the --chap and the --hap--snp were used together.

--window 4 command was used to include all 4 SNPs in the haplotype

The full line of command used in this thesis for haplotype analysis is:

Plink --bfile mydata --1 --hap-window 4 --hap-assoc

The output file generated by the program after this calculation is saved as plink.hap

6.4.1.4 Gene x Environment analysis

GxE analysis was performed with logistic regression test using HapStat version 3.0 (Lin et al., 2008). Hapstat is a user-friendly software that allows testing of single markers and haplotype-disease association. The haplotype association is calculated by maximizing the observed data likelihood that accounts for phase uncertainty and study design (Lin et al., 2008).

Environmental variable entered was:

- cannabis use (variable modeled as binary trait: yes/no answer)

Genetic variables entered were:

- rs6269
- rs4633
- rs4818
- rs4680
- LPS haplotype chosen a priori.

For the exploratory analysis the environmental variables entered were:

- frequency of cannabis use (variable modeled as binary trait: high frequency users Vs low frequency use)
- Age of first use (variable modeled as binary trait: before and after the age of 15yrs)

Disease Status was always modeled as binary trait: psychotic subjects Vs non-psychotic subjects)

6.4.1.4.1 Cannabis use data

Cannabis use data were collected as part of the Cannabis Experience Questionnaire explained in detail in chapter 4 and chapter 7 of this thesis.

Subjects were asked if they ever used cannabis, frequency of use and age of first use. All data are retrospectively based.

For the GxE analysis I used the data on cannabis use (yes/no) and excluded all individuals with no data. For the exploratory analysis I used the frequency of cannabis use modeled as before or after 15 years of age. This cut off is in line with the literature on the subject (Arsenault et al., 2002) (Stefanis et al., 2004) and offers an arbitrary mark of adolescence. Frequency of use was classified as frequent VS non frequency, where the first refers to use of 3 or more times per week.

6.4.1.5 Bonferroni Correction for multiple testing

Bonferroni conservative correction for multiple testing was applied after allelic and genotype association analysis. For the logistic regression, the markers analysed were 7, Bonferroni corrected p-value calculated as 0.05/7 would be 0.007.

In the tables, Bonferroni corrected p-value is reported as χ^2 p-value * number of tests performed.

6.5 Results

6.5.1 Hardy Weinberg Equilibrium Test

Hardy Weinberg Equilibrium test was examined in non psychotic control groups of the GAP Study Caucasian group, the GAP Study Black group and the PICOS Study separately. The PICOS group was only genotyped for rs4680. In every statistical analysis performed in this thesis, they were analysed separately as 3 distinct populations. Deviation from equilibrium was checked for a total of 7 SNPs namely rs6269, rs4633, rs4818, rs4680, rs2075507, rs165599 and rs737865 within the COMT gene. One SNP namely rs165599 was found to differ from Hardy Weinberg expected values in the GAP Caucasian group: p-value=0.048 (Complementary Table C.7), but was still included in the subsequent analysis after accounting for multiple testing correction (Bonferroni multiple testing threshold is 0.05/7=0.007).

In the GAP Black group none of the markers failed the HWE test (Complementary Table C.8).

The PICOS Study sample was only genotyped for rs4680 which was found to be in equilibrium after performing HWE test (p-value=0.45).

6.5.2 Association between allelic and genotype variation at each locus within the COMT gene and Psychosis

The main effect of each SNP at each locus within the COMT gene on psychosis was tested with a χ^2 test in the 3 study samples separately.

No association was found in the GAP Study sample Caucasian or Black group for any of the markers analysed within the COMT gene.

Moreover, no association was found with rs4680 and psychosis in the PICOS Study sample.

Chi square and p-values could not be calculated for rs737865, rs6269, rs4818 and rs2075507 in the GAP Caucasian group for genotype association because 1 genotype at each locus, G/G, G/G, G/G and G/G respectively had a very low frequency in the psychotic or non psychotic group of subjects (Table 6.5).

Frequency of 1 genotype (rs737865 G/G) was also too low for chi square and p-value calculation in the GAP Black group (Table 6.6).

All results are shown in Tables 6.2 to 6.6.

As mentioned in section 6.4.1.4 Bonferroni correction in this analysis is applied by dividing p-values by number of SNPs analysed: 0.05/7=0.007. This is the threshold for significance in this particular test. No association however was observed, Bonferroni correction is therefore not needed.

Frequencies of minor alleles are, for some of the COMT markers analysed, differ to the ones reported by NCBI that can be seen in Table 6.1. For example rs6269 has a MAF of 0.29 in the non psychotic Caucasian group, and of 0.43 in the non psychotic Black group, whereas NCBI reports a MAF of 0.37. MAF of rs4680 seem to differ between the 2 Caucasian groups analysed in this study, the GAP and the PICOS, 0.38 and 0.46 respectively. NCBI reports a MAF of 0.39 for rs4680, thus closer to the one observed in the GAP Caucasian group. This difference in values could reflect ethnic differences present within European populations of different countries or indeed a reflection of low numbers in the population analysed. In the presence of a positive association, this observation would be more important, because, as observed in chapter 5 of this thesis, it could lead to statistical false positive. In this case, however, no association was found and it is unlikely a false negative result, even in the presence of many limitations, discussed further in the conclusion paragraph of this chapter.

TABLE 6.2: Association between allelic variation at each locus within COMT and Psychosis in the GAP Caucasian sample

Gene	rs number	Minor Allele/ Other allele	psychotic participants (MAF)	psychotic participants (N)	non psychotic participants (MAF)	non psychotic participants (N)[(a)]	CHISQ (DF=1)*	P-Value	OR
COMT	rs737865	G/A	0.16	155	0.12	43	1.06	0.303	1.46
COMT	rs6269	G/A	0.38	168	0.29	41	1.94	0.164	1.45
COMT	rs4633	T/C	0.34	148	0.41	43	1.26	0.262	0.75
COMT	rs4818	G/C	0.20	167	0.18	41	0.09	0.764	1.10
COMT	rS4680	A/G	0.34	154	0.38	32	0.33	0.567	0.85
COMT	rs2075507	G/A	0.34	150	0.38	23	0.30	0.585	0.86
COMT	rs165599	A/G	0.38	166	0.4	41	0.07	0.77	0.93

MAF= Minor allele frequency
OR=Odd Ratio
(a)= number of participants (Psychotic and non psychotic) for genotype groups at each locus
* Degrees of freedom=1

TABLE 6.3: Association between allelic variation at each locus within COMT and Psychosis in the GAP Black sample

Gene	rs number	Minor Allele/ Other allele	psychotic participants (MAF)	psychotic participants (N)	non psychotic participants (MAF)	non psychotic participants (N)[(a)]	CHISQ (DF=1)*	P-Value	OR
COMT	rs737865	G/A	0.30	105	0.28	89	0.17	0.680	1.10
COMT	rs6269	G/A	0.40	105	0.43	88	0.38	0.539	0.88
COMT	rs4633	T/C	0.48	97	0.46	91	0.21	0.650	1.10
COMT	rs4818	G/C	0.37	109	0.39	89	0.20	0.658	0.91
COMT	rs4680	A/G	0.51	92	0.46	72	0.72	0.397	1.21
COMT	rs2075507	G/A	0.39	95	0.33	71	1.42	0.233	1.32
COMT	rs165599	A/G	0.33	109	0.34	94	0.04	0.82	0.95

MAF= Minor allele frequency
OR=Odd Ratio
(a)= number of participants (Psychotic and non psychotic) for genotype groups at each locus
* Degrees of freedom=1

TABLE 6.4: Association between allelic variation at rs4680 locus within COMT and Psychosis in the PICOS Study sample

Population	Gene	rs number	Minor Allele/ Other allele	psychotic participants (MAF)	psychotic participants (N)[(a)]	non psychotic participants (MAF)	non psychotic participants (N)[(a)]	CHISQ (DF=1)*	P-Value	OR
PICOS	COMT	rs4680	A/G	0.44	302	0.46	476	0.34	0.55	0.94

MAF= Minor allele frequency
OR=Odd Ratio
(a)= number of participants (Psychotic and non psychotic) for genotype groups at each locus
* Degrees of freedom=1

TABLE 6.5: Association between genotype variation at each locus within COMT and Psychosis in the GAP Caucasian sample

Gene	rs number	Minor Allele/ Other allele	psychotic participants (a)	psychotic participants (N)	non psychotic participants (a)	non psychotic participants (N)[(b)]	CHISQ (DF=1)*	P-Value
COMT	rs737865	G/A	7/36/112	155	1\8\34	43	NA	NA
COMT	rs6269	G/A	26/74/68	168	4/16/21	41	NA	NA
COMT	rs4633	T/C	17/67/64	148	8/19/16	43	1.59	0.452
COMT	rs4818	G/C	9/48/110	167	0/15/26	41	NA	NA
COMT	rs4680	A/G	15/74/65	154	5/14/13	32	0.97	0.615
COMT	rs2075507	G/A	18/67/65	150	4/17/12	23	NA	NA
COMT	rs165599	A/G	31/66/69	166	10/13/18	41	1.143	0.5645

(a)= number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele
(b)= number of participants (Psychotic and non psychotic) for genotype groups at each locus
* Degrees of freedom=2

TABLE 6.6: Association between genotype variation at each locus within COMT and Psychosis in the GAP Black sample

Gene	rs number	Minor Allele/ Other allele	psychotic participants (a)	psychotic participants (N)	non psychotic participants (a)	non psychotic participants (N)[(b)]	CHISQ (DF=1)*	P-Value
COMT	rs6269	G/A	18/47/40	105	19/37/32	88	0.61	0.736
COMT	rs4633	T/C	24/45/28	97	22/39/30	91	0.39	0.821
COMT	rs4818	G/C	18/45/46	109	15/40/34	89	0.35	0.839
COMT	rs4680	A/G	24/45/23	92	15/36/21	72	0.74	0.691
COMT	rs2075507	G/A	10/55/30	95	11/25/35	71	8.39	0.015
COMT	rs165599	A/G	11/50/48	109	14/36/44	94	1.714	0.4244
COMT	rs737865	G/A	9/45/51	105	4/42/43	89	NA	NA

(a)= number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele
(b)= number of participants (Psychotic and non psychotic) for genotype groups at each locus
* Degrees of freedom=2

6.5.3 Haplotype analysis

I decided to further explore the data by performing haplotype analysis testing the main effect of the LPS, a priori set as the risk haplotype, on psychosis. I calculated Linkage Disequilibrium (LD) for the SNPs within the COMT gene using haploview and carried out the association analysis using PLINK version 1.7 (Purcell et al., 2007).

All analyses were carried out in the GAP Study sample Caucasian and Black group separately.

For a better understanding of the level of correlation between the 6 COMT SNPs in the populations examined in this chapter, I calculated the D' and the r2 values with haploview (Table 6.7, Table 6.8). D' and r2 scores for the 4 SNPs of the LPS haplotype (rs6269, rs4633, rs4818 and rs4680)are displayed in figure 6.3 and 6.4. LD blocks plots with r2 values in the Caucasian and the Black populations are shown in Figures 6.1 and 6.2.

Figure 6.1 Linkage Disequilibrium Plot computed with markers within the COMT gene in the GAP Caucasian sample

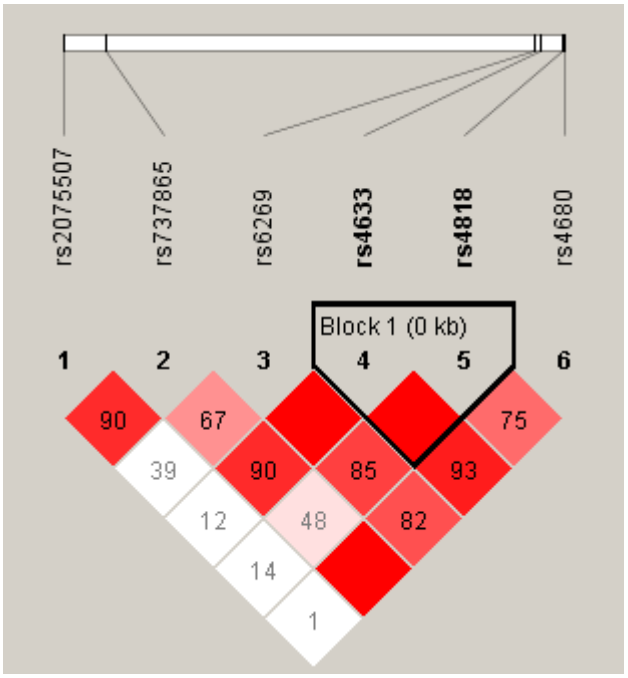


Figure 6.2 Linkage Disequilibrium Plot computed with markers within the COMT gene in the GAP Black sample

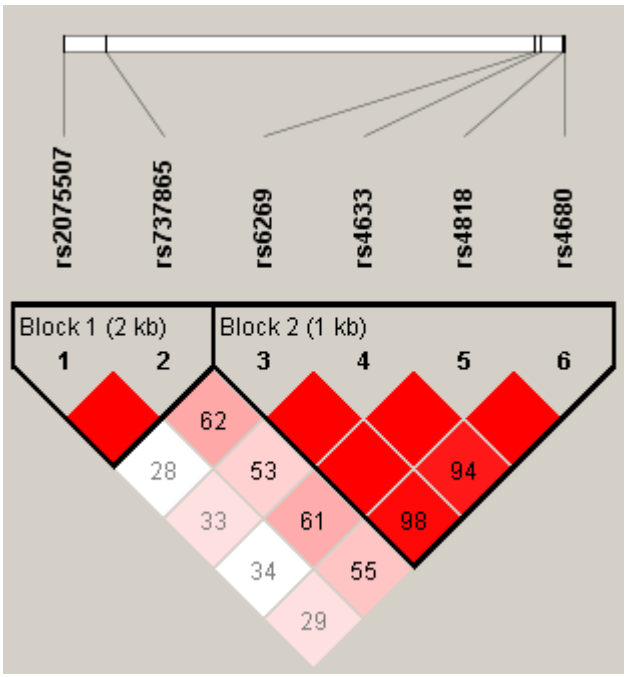


Figure 6.3 Linkage Disequilibrium Plot computed of the LPS haplotype within the COMT gene in the GAP Caucasian sample

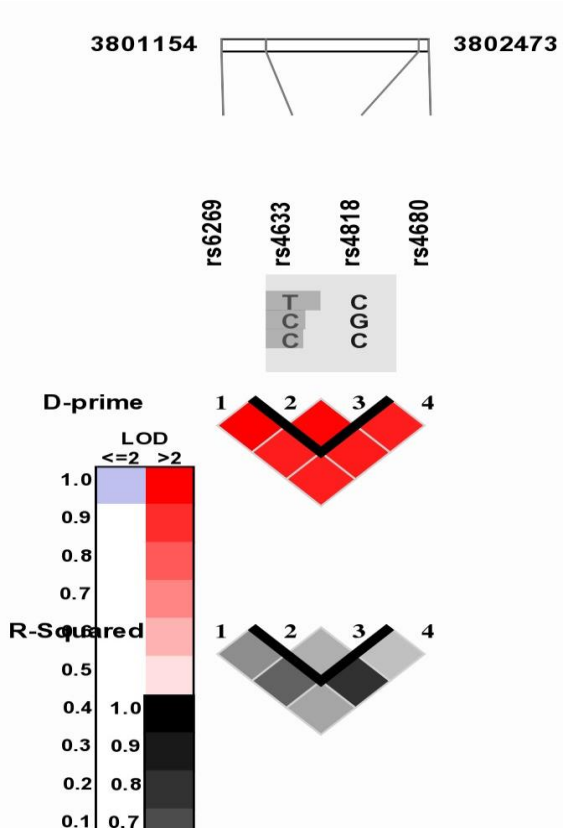


Table 6.7 D' and r2 values calculated with Haploview in the GAP Caucasian sample (shown in bolt markers with higher D' and r2 scores further discussed in the conclusion paragraph of this chapter)

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs6269	rs4633	1	0.338
rs6269	rs4818	0.856	0.327
rs6269	rs4680	0.893	0.227
rs4633	rs4818	0.942	0.137
rs4633	**rs4680**	**0.96**	**0.774**
rs4818	rs4680	0.841	0.09

Figure 6.4 Linkage Disequilibrium Plot computed of the LPS haplotype within the COMT gene in the GAP Black sample

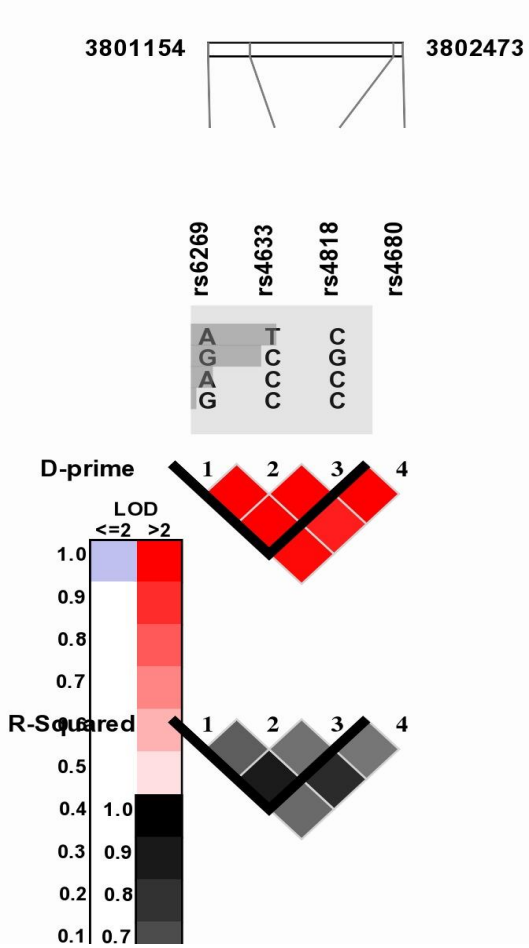


Table 6.8 D' and r2 values calculated with Haploview in the GAP Black sample (shown in bolt markers with higher D' and r2 scores further discussed in the conclusion paragraph of this chapter)

MARKER 1	MARKER 2	D' VALUE	r2 VALUE
rs6269	rs4633	1	0.629
rs6269	rs4818	1	0.893
rs6269	rs4680	0.98	0.582
rs4633	rs4818	1	0.553
rs4633	**rs4680**	**0.933**	**0.828**
rs4818	rs4680	1	0.536

When analysing haplotypes, it is important to interpret results in light of D' and r2 scores. D' values refer to the rate at which analysed SNPs tend to be co-transmitted, the higher the D' score, the lower the probability of historical recombination. D' scores though tend to be higher in the presence of a small sample, like the one analysed in this chapter. It is generally a very informative value, but in this case, sample size plays a big role in its calculation. R2 values represent the degree of independence between SNPs, the higher the value, the less independent from each other 2 signals will be. It is therefore a very useful score to take into consideration when interpreting results from association studies. The LPS haplotype accounts for more than 90% of genetic variation at that site in the population, as reported by Diatchenko and colleagues (Diatchenko et al., 2006). The same frequencies can be seen in both the GAP Caucasian and the GAP Black group analysed in this chapter (Tables 6.9 and 6.10). D' scores are therefore high and close to 1 within the 4 SNPs that make the LPS/APS/HPS haplotypes (Tables 6.7 and 6.8). R2 values are higher between rs4633 and rs4680 in both the GAP Caucasian and Black group r2=0.77 and r2=0.82 respectively (Tables 6.7 and 6.8). These 2 SNPs therefore seem to act as proxies in the sample analysed, which indicates that we are not in the presence of independent signals from these 3 markers.

The LPS susceptibility haplotype consists of 4 SNPs within the COMT gene: rs6269, rs4633, rs4818 and rs4680 (GCGG) was analyzed against all other haplotypes in a window 4 type analysis in PLINK (Purcell et al., 2007) in the GAP Caucasian and the GAP Black group separately.

No main effect of LPS on risk of psychosis was found in either group: OMNIBUS p-value=0.59 LPS P-value=0.61 and OMNIBUS p-value=0.7 LPS P-value=0.5 respectively (Tables 6.9 and 6.10).

These results show no main effect of the LPS or any other haplotype on risk of psychosis. Although both study samples are small, type 2 error can be safely excluded.

TABLE 6.9: Main effect of haplotype on risk of psychosis in the GAP Caucasian sample

SNPS	HAPLOTYPE	HAPLOTYPE NAME	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value
rs6269|rs4633|rs4818|rs4680	OMNIBUS		NA	NA	3.738	5	0.5878
rs6269|rs4633|rs4818|rs4680	**GCGG**	**LPS**	**0.1822**	**0.1581**	**0.2586**	**1**	**0.6111**
rs6269|rs4633|rs4818|rs4680	ATCA	APS	0.3155	0.37	0.8793	1	0.3484
rs6269|rs4633|rs4818|rs4680	ACGG		0.01186	0.02702	1.022	1	0.3121
rs6269|rs4633|rs4818|rs4680	ATCG		0.05103	0.05261	0.00333	1	0.954
rs6269|rs4633|rs4818|rs4680	GCCG		0.1851	0.1161	2.176	1	0.1401
rs6269|rs4633|rs4818|rs4680	ACCG	HPS	0.2543	0.2762	0.1615	1	0.6878

TABLE6.10: Main effect of haplotype on risk of psychosis in the GAP Black sample

SNPS	HAPLOTYPE	HAPLOTYPE NAME	Frequency in Psychotics	Frequency in non psychotics	CHISQ	DF	P-value
rs6269|rs4633|rs4818|rs4680	OMNIBUS		NA	NA	2.992	5	0.7012
rs6269|rs4633|rs4818|rs4680	GCGG	LPS	0.365	0.3978	0.4448	1	0.5048
rs6269|rs4633|rs4818|rs4680	ACCA		0.01796	0.002355	2.245	1	0.134
rs6269|rs4633|rs4818|rs4680	ATCA	APS	0.4732	0.4453	0.3052	1	0.5806
rs6269|rs4633|rs4818|rs4680	ATCG		0.01224	0.01942	0.3258	1	0.5681
rs6269|rs4633|rs4818|rs4680	GCCG		0.02498	0.02525	0.000291	1	0.9864
rs6269|rs4633|rs4818|rs4680	ACCG	HPS	0.1066	0.1098	0.01066	1	0.9178

6.5.4 Gene x Environment Analysis

In order to explore the correlation between the LPS haplotype and cannabis use, I performed a logistic regression test. Firstly I tested all markers forming the LPS haplotype, namely rs6269, rs4633, rs4818 and rs4680 and then the a priori hypothesis: the LPS haplotype under additive model.

There was no association between any of the 4 markers within the COMT gene or the LPS haplotype and cannabis use in either of the 2 study samples (Tables 6.11). Variations within the COMT gene analysed in this study did not seem to have any effect on cannabis use.

Table 6.11 Correlation between cannabis use and COMT

POPULATION	GENE	MARKER	P-VALUE	STANDARD ERROR	Z SCORE
Caucasian	COMT	rs6269	0.1	0.2	-1.5
Caucasian	COMT	rs4633	0.8	0.2	0.2
Caucasian	COMT	rs4818	0.07	0.2	-1.7
Caucasian	COMT	rs4680	0.2	0.2	1.2
Caucasian	COMT	LPS	0.06	0.2	1.8
Black	COMT	rs6269	0.06	0.2	-2.1
Black	COMT	rs4633	0.1	0.2	-1.6
Black	COMT	rs4818	0.06	0.2	-1.8
Black	COMT	rs4680	0.3	0.2	-1
Black	COMT	LPS	0.06	0.23	1.9

Gene x Environment interaction was calculated with logistic regression test under additive model. No interaction was found between any of the COMT markers analysed in this study or the LPS haplotype and cannabis use in either of the GAP Study sample groups (Table 6.12).

Table 6.12 Gene x Environment interaction between the COMT gene and Cannabis use

POPULATION	GENE	MARKER	P-VALUE	STANDARD ERROR	Z SCORE
Caucasian	COMT	rs6269	0.58	0.23	-0.55
Caucasian	COMT	rs4633	0.5	0.26	-0.6
Caucasian	COMT	rs4818	0.09	0.3	-1.6
Caucasian	COMT	rs4680	0.77	0.25	0.28
Caucasian	COMT	LPS	0.09	0.3	1.6
Black	COMT	rs6269	0.06	0.3	-1.8
Black	COMT	rs4633	0.1	0.3	1.3
Black	COMT	rs4818	0.3	0.3	-1.03
Black	COMT	rs4680	0.5	0.3	0.6
Black	COMT	LPS	0.3	0.3	1

6.5.5 Exploratory analysis

An explorative analysis to test whether use of cannabis during adolescence could have a higher impact in the interaction, data on adolescent cannabis use was modeled as binary trait and divided in two groups: 1. first use before the age of 15; 2. first use at or after the age of 15. Non cannabis users were excluded from the analysis.

Analysis could only be carried out in the GAP group including all ethnic groups due to limited number of adolescent cannabis use available. The additive model for the LPS was the *a priori* hypothesis and the only tested in this analysis.

Results showed no interaction between the LPS haplotype and age of first use in Caucasian or Black populations (p-values of 0.3 and 0.09 respectively).

6.6 Summary of results

In this chapter I have tested the main effect of 7 markers within the COMT gene, namely rs6269, rs4633, rs4818, rs4680, rs2075507, rs165599 and rs737865 and 3 haplotypes formed by 4 of the markers (rs6269, rs4633, rs4818, rs4680), namely the LPS, APS and HPS.

- Results showed no presence of main effect on psychosis at any of the loci nor the haplotypes.

- Exploring correlation between each of the markers, the LPS (a priori hypothesis) and lifetime cannabis use showed that genetic variants examined did not have any effect on cannabis use.
- Genetic x environment analysis also showed no interaction. Moreover, exploratory analysis on interaction between use of cannabis before or after adolescence and the LPS haplotype on risk of psychosis also returned no positive results.

6.7 Conclusions

The COMT gene is among those most studied in association with psychosis, also because of its reported interaction with cannabis use. It seems to be a good candidate because of its role in dopamine catabolism in the prefrontal cortex. Indeed many studies have shown positive association, even stronger when cannabis use started before adolescence or when the type of cannabis smoked was of the strongest (Skunk, Sinsemilla). Other studies however, have failed to identify any association. Findings of the association studies therefore remain conflicting for the COMT gene, whereas the most recent findings of the GWAS are very clear and do not mention it among the most promising genes for schizophrenia. The role of COMT though may be more apparent in the presence of a well characterised cohort also offering environmental data such as drug abuse. In this chapter I have tried to replicate findings of interplay between lifetime use of cannabis and psychosis. Results were unanimously negative. It is very important though to look at these results in light of many limitations present in this study. First and foremost the sample size is small to detect an interaction of polymorphisms that may only account for a very little effect in the aetiology of a complex disorder. To detect an effect of a common polymorphism (MAF>5%) on a common disorder, numbers should equal those of the latest GWAS performed. This study also analysed environmental risks, adding more power to the analysis, but a sample size of less than 200 cases and 100 controls has no power to detect any interaction or association unless in the presence of a rare variant (MAF<1%) with a substantial effect size. As shown in the power analysis (Materials and Methods chapter), in order to achieve 80% of power for detecting a true association between a frequent allele (MAF>0.5) with an effect size of 1.2 for a population risk of disease of 1%, there is the need of 6000 case-controls pairs. Adding environmental effects to the model to calculate a gene x environment interaction, assuming an exposure of 40% and an effect size of 1.2, there is the need of 20,000 case-control pairs. Combining the two groups, Caucasian and Black within the GAP study sample would have added no power to the analysis as the bias of ethnic stratification in such different populations would have been too great. Of note there is also the possible discrepancy between self-reported frequency/amount of cannabis used and the real figures. Furthermore, in this study, all cannabis data are collected retrospectively. This may pose a potential bias within the group of psychotic subjects due to possible cognitive impairments. One of the future directions would be to certainly

increase the sample size within both groups of the GAP study sample. This would ensure more power to the analyses, therefore increasing accuracy. One of the strengths of the GAP study is the recruitment of ethnically different populations at the same time; this allows analysing the comparison of findings in different groups. They will however have to be of a bigger number in future studies. Overall, from the analyses performed it can be concluded that the markers and the haplotype within the COMT gene in this chapter do not exert an effect on psychosis, or lifetime cannabis use, nor they seem to interact with cannabis on the increase of risk of psychosis.

CHAPTER 7

RESULTS

Psychological Experiences in Cannabis Use: The Mediating role of the COMT gene

7.1 Background information

Cannabis is one of the most abused drugs among young people. Many of them report a calming, pleasurable experience, but many report feeling anxious and paranoid. It has long being discussed whether some personality trait leads to consumption of cannabis in an attempt to self medicate (Chackroun et al., 2004) (Howes et al., 2004) (Howes and Kapur, 2009). Impulsivity and schizotipy both seem to play a role in initiating drug abuse behaviour. Impulsivity is characterised by a motor component and an attention component (as reviewed in Barkus et al., 2006) and it has been associated with cannabis use but findings are conflicting (Boden et al., 2006) (Adams et al., 2003) (Crawford et al., 2003). Schizotypy is a trait that is normally distributed in the general population. It is characterised by an unusual belief system, anxiety, depression, social withdrawn and attenuated psychotic symptoms (Barkus et al., 2008). Whether individuals with schizotypy use more cannabis, use it to self medicate and will develop schizophrenia later in life has long been studied but with conflicting conclusions. Henquet et al, showed that subjects with psychosis liability were more likely to suffer from long term effect of cannabis use than those who did not (Henquet et al., 2005b). Assessing psychological experiences during and after drug self administration is thus very important. This chapter is based on an adapted version of the Cannabis Experience Questionnaire (CAQ) developed by Barkus et al. to test the likelihood of non psychotic subjects who scored high in schizotypal personality, of experiencing more psychosis like symptoms upon use of cannabis (Barkus et al., 2006). They found that indeed, subjects that scored high in the schizotypal personality test were also more likely to experience more psychotic like experience and unpleasant effects after smoking (Barkus et al., 2006). Findings later confirmed by a follow up study in 2008 (Stirling et al., 2008). Furthermore, Henquet et al., in a double blind, placebo controlled cross over study with psychotic patients, relatives and healthy controls showed that carriers of the Val158Met allele of the COMT gene were more sensitive to psychotic experience induced by the compound and to worst results in memory and attention tasks; the same subjects showed prior psychometric psychosis liability (Henquet et al., 2006). The COMT gene may therefore play a role in the complex puzzle of psychosis liability, cannabis use, pleasurable VS unpleasant experiences upon cannabis use and development of schizophrenia later in life.

7.2 Hypothesis under investigation

In this chapter I will analyse self reported experiences during and after cannabis consumption. I will reduce the items to be analysed by combining them using Principal Component Analysis. I will then explore the relationship between cannabis use with the perceived experiences. I hypothesise that:

1. psychotic subjects will experience more anxiety/paranoia or perception abnormality response
2. healthy subjects will experience more pleasurable response

I will also explore the role of the rs4680 polymorphism and the LPS haplotype within the COMT gene in mediating the role of cannabis. I hypothesise that:

1. subjects with more copies of the risk Val allele will report more anxiety or hallucinatory response
2. people with more copies of the LPS haplotype will report more anxiety or hallucinatory response

I will conduct further analysis to elucidate the role of a) frequency of drug abuse b) age of first use c) type of cannabis used. I hypothesise that:

1. heavier consumers are those who experience more pleasurable response
2. having more copies of the risk Val allele and using cannabis more often predisposes subjects to experience more anxiety/paranoia and or perception abnormalities
3. having more copies of the risk LPS haplotype and using cannabis more often predisposes subjects to experience more anxiety/paranoia and or perception abnormalities

7.3 Statistical analysis

For this chapter I only used the GAP Study sample, Caucasian and Black group. As this chapter focuses on the self reported experiences during and after cannabis consumption, the population used includes cannabis smokers only (present and past) from the case and the control group. Non cannabis smokers are excluded. Moreover, the population includes subjects from both ethnic backgrounds analysed together, as it was not possible to stratify for ethnicity due to low number of patients with cannabis data.

All analysis refers to the Caucasian and Black group together as the GAP Study sample.

The total sample size used for this chapter is summarised in Table 7.1.

In this chapter statistical tests performed were:

- Principal Component Analysis (SPSS version IBM 19)

- Linear regression test for association analysis
- Bonferroni correction for multiple testing

Table 7.1 Summary of sample selection

ETHNICITY	STATUS	CANNABIS EXPERIENCES INFORMATION	LPS INFORMATION	rs4680 INFORMATION
Black	case	34	28	29
Black	control	34	21	16
Caucasian	case	26	20	12
Caucasian	control	20	12	9

7.3.1 The Cannabis Experience Questionnaire

Cannabis data were obtained by a screening questionnaire used for both cases and controls. The questionnaire is the revised version of the Cannabis Experience Questionnaire (Barkus et al., 2006) and it includes date of first use, preferred mode of use, frequency as well as data on self reported feelings before and after consumption (Appendix). The Cannabis experience questionnaire contains 19 items with multiple choice answers as well as an open answer. From item 14 onwards it collects perceived experiences while smoking and after. It also establishes type of cannabis used and quantity smoked during adolescence and adulthood. The questionnaire also collects information on other drugs used in combination or during the same period of time as cannabis consumption, this helps to correct for possible confounders.

For the factor analysis reported in this chapter, section 15.14 and 15.15 of the cannabis questionnaire were used. Section 15.14 addresses the question of self reported experiences during cannabis consumption whereas section 15.15 refers to experience occurred after the effect of cannabis has worn off. Participants are given a list of experiences (9 for section 15.14 and 5 for section 15.15) for which they have to rate the frequency (a) rarely or never; b) from time to time; c) sometimes; d) more often than not; e) almost always) and how they felt about it (a) good; b) bad and c) neutral).

The Cannabis Experience Questionnaire was administered to patients and control by the group of clinical researchers of the GAP team. I was part of this group for the whole first year of my PhD, before starting laboratory work. I was assigned to the group of researchers recruiting non psychotic volunteers. Subjects included in this chapter were also, but not all, recruited by the team of which I was part. My contribution can be estimated to be of a total of 40% of the non psychotic population included in this chapter.

7.3.2 Principal Component Analysis

Principal Component Analysis (PCA) with Varimax rotation was conducted using IBM SPSS version 19. PCA is a factorial technique that allows analysing correlation among variables which occurs when they measure the same underlying dimension. It is possible therefore to group correlated variables into factors independent of each other. I will use the term factor analysis to describe PCA and factors to refer to components. Factors were extracted using PCA and rotated using Varimax with Kaiser Normalisation. Rotation maximises the load of each variable into a factor while, at the same time, minimising the load on the other factors. Orthogonal rotation was chosen because I expected the factors to be independent from each other. Scores were calculated under the Anderson-Rubin method. Factors can only be calculated if the percentage of data missing is low in any of the variables. Low frequency variables 15.14 g) hearing voices and 15.15 b) being suspicious without reason were excluded from the analysis. An experience-factor loading cut off point of >0.4 was used to interpret the factors. Finally, Keiser-Meyer-Olkin test and Barlett's test of sphericity were used to assess adequacy of sample size.

Variables included were from section 15.14 and 15.15 of the adapted Cannabis Experience Questionnaire as follow:

- Section 15.14 (self reported experiences during cannabis consumption)

a Fearful
b Feel like going crazy/mad
c Nervy
d Suspicious
e Feeling happy
f Full of plans/ideas
g Able to understand the world better
h Seeing visions

- Section 15.15 (self reported experiences after cannabis consumption)

a Not wanting to do anything
b Slowed down thinking
c Difficulty in concentrating
d Not able to think clearly

Subjects were also asked to rate the extent to which they thought the experience was enjoyable, but this measure was not used for this analysis.

7.3.3 Association analysis

Stepwise linear regression analysis was carried out to test association between each cannabis-response factor score, rs4680 and the LPS haplotype within COMT, and frequency of cannabis use. I used SPSS version 19.

For the main correlation analyses each factor score was entered in the regression models as a dependent variable. The following variables were entered as independent variables:

- Disease status: psychotic subjects VS non psychotic subjects (variable modelled as binary trait: 1,0)
- Rs4680 genotype was coded as number of copies (0,1,2) of the risk Val allele.
- LPS the *a priori* risk haplotype (variable was coded in number of copies of LPS haplotype for each subject: values are 0, 1 and 2). Haplotype pairs for each subject were obtained using PHASE package for Windows version 2.1 (Stephens et al., 2001) and only pairs that exceeded probability of 80% were considered.
- Frequency of cannabis use (data were coded in a binary variable: people reporting to have used cannabis between less than once a week and few times per year were grouped as light smokers – group 1 -; people reporting to have used cannabis twice a week or more were grouped as heavy smokers – group 2-).
- Age of first cannabis use (data were coded in a binary variable: before or after the age of 15)
- Finally, type of cannabis used (dichotomised as strong Vs non-strong) was an exploratory variable added to the model. Strong type of cannabis refers to the hypothetical higher content of Δ9-THC, the psychoactive component; and lower concentration of CBD, which is thought to exert anti-psychotic activity.

7.3.4 Bonferroni Correction for multiple testing

Bonferroni conservative correction for multiple testing was applied after logistic regression analysis. Factor analysed were 4, Bonferroni corrected p-value calculated as 0.05/4 would be 0.01. In the tables, Bonferroni corrected p-value is reported as x2 p-value*number of tests performed.

7.4 Results

7.4.1 Principal Component Analysis

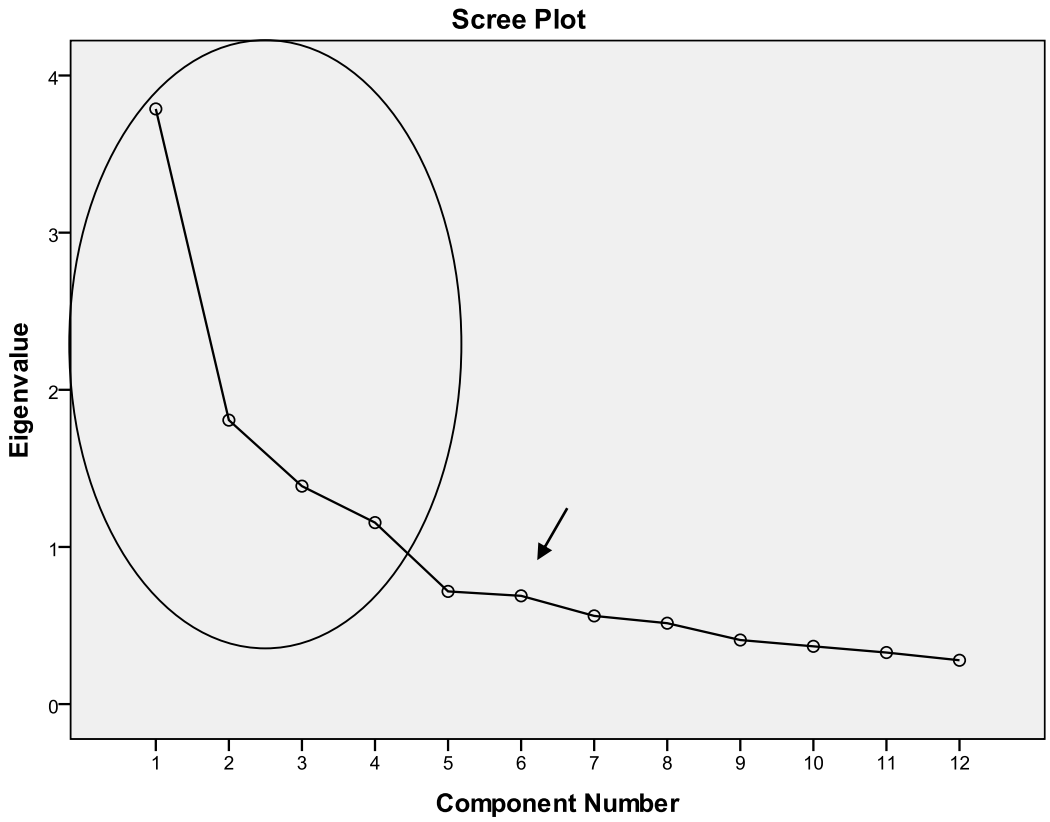
Results showed that all 14 variables could be grouped by 4 independent dimensions or factors, each mainly exploring a different response to cannabis use.

Results of the factorial analysis are shown in Table 7.2 and Figure 7.1

Table 7.2 Factors loading obtained from the Principal Component Analysis conducted on sections 15.14 and 15.15 of the Cannabis Experience Questionnaire (CEQ). Samples are of the GAP group and all ethnicities are included.

	Factors			
CEQ Variables	Anxiety/Paranoia response **FACTOR 1**	Inhibitory/depressive response **FACTOR 2**	Pleasurable response **FACTOR 3**	Perception abnormalities response **FACTOR 4**
fearful	**.676**	.150	-.025	-.034
feel like going crazy	**.436**	.185	.090	.410
nervy	**.756**	.164	.035	.057
suspicious	**.709**	.227	.027	.193
not wanting to do anything	.211	**.572**	.088	.079
slowed down thinking	.218	**.707**	.059	.202
difficulty in concentrating	.255	**.733**	.090	.091
feeling happy	-.231	.109	**.431**	-.022
Full of plans or ideas	.187	.133	**.816**	.082
able to understand world better	.077	.001	**.465**	.142
hearing voices	.293	.116	.101	**.566**
seeing visions	.020	.155	.101	**.565**

Figure 7.1 Scree plot with results of the Factor Analysis



As it can be seen from the plot highlighted inside of the circle, there are 4 factors clearly visible before the curve starts its plateau. However, after factor 6 (marked with an arrow), another plateau is visible. I could therefore choose to run the factorial analysis force including 6 factors, but because of the small sample size and possible error, I decided that it was safe to assume the Kaiser's criterion and only retained the 4 factors illustrated in table 7.2.

The 4 factors clearly describe a range of different dimensions of experiences. Experiences during and after consumption of cannabis were pooled together in this analysis in an attempt to get a better idea of the whole experience of the use of the drug. As suggested by Barkus et al., after use experiences appear to identify the "amotivational syndrome" frequently reported by people with daily use of cannabis (Barkus et al., 2006). Analysing self reported experiences during and after abuse also gives a wider insight into the motivation behind perpetuation of the behaviour. If a subject experiences pleasurable effects during consumption and no "amotivational syndrome" afterwards, he or she is more likely to continue abusing the drug than a subject experiencing paranoia or anxiety upon use and amotivation afterwards, especially if this interferes with daily tasks. Pooled experience factor analysis show that each factor can be related to features observed in psychotic disorder.

Factor 1 includes items: a) fearful, b) feel like going crazy, c) nervy and d) suspicious, a group of experiences that refers to an anxiety or paranoid response to cannabis consumption.

Factor 2 includes: a) not wanting to do anything, b) slowed down thinking and c) difficulty in concentrating thus exploring an inhibitory and depressive response to cannabis consumption.

Factor 3 includes: a) feeling happy, b) full of plans and ideas, c) able to understand the world better and seems to highlight the most pleasurable response to cannabis consumption.

Factor 4 includes: a) hearing voices and b) seeing visions and seems to refer to perception abnormalities in response to cannabis consumption.

Keiser-Meyer-Olkin sample adequacy value of 0.754, exceeded the recommended of 0.5 and Barlett's test reached significance with a p-value=0.004. It is therefore safe to assume that, in light of sample adequacy, that the clear load of the variables into 4 factors and their independence between each other, the results of the analysis are an indication of the range of experiences reported in the questionnaire.

7.4.2 Association analysis

I decided to further explore the results of the factorial analysis by analysing factors correlation with psychosis and cannabis use. I firstly analysed the correlation between experiences (factor1-4), disease status and genetic data (copies of the Val allele at rs4680 locus and copies of the LPS haplotype).

I subsequently run exploratory analyses by adding 3 further variables to the model: frequency of cannabis use (dichotomised high Vs low), age of first use (dichotomised before or after 15 years of age) and type of cannabis use (dichotomised Stong Vs weak).

Results of the linear regression analysis showed no correlation between disease status and any of the factors (Table 7.3). Moreover, it showed no correlation between number of risk allele Val copies at the rs4680 locus and any of the factors (Table 7.4). In this sample, being a patient or a control did not predict the type of experiences the subject was going to have upon cannabis consumption. Furthermore, having more or less copies of the Val risk allele at the rs4680 locus did not make subjects more likely to have more or less pleasurable experiences with cannabis use.

Copies of the LPS haplotype were however positively associated with the inhibitory/depressive response (Factor 2) (p-value=0.02). Having less or no copies of the LPS risk haplotype predisposes individuals to experience a more inhibitory/depressive response upon consumption of cannabis. Results also showed that having more copies of the LPS haplotype predisposes individuals to experience more perception abnormalities (Factor 4) upon cannabis consumption (p-value=0.04). This however did not retain significance after Bonferroni correction for multiple testing (adjusted p-value=0.08 and 0.16 respectively) (Table 7.5), it is therefore not a true association. Associations were independent of disease status (p-value=0.4 and p-value=0.8 respectively) and ethnicity (p-value=0.36 and p-value=0.53 respectively).

64.8% of our sample of users reported they had used cannabis 3 or more times per week (high frequency users) and 35.2% reported to have used it less than twice a week/sometimes/once a month (low frequency users). 48% started to use cannabis before the age of 15, whereas 52% started afterwards.

Association analysis showed that frequency of cannabis use was associated with Factor 1 (Table 7.6). Subjects that used cannabis 3 or more times per week, tended to have more anxiety and paranoia response to the drug (adjusted p-value=0.004). This suggests that people that use cannabis more than 3 times per week are more likely to respond with anxiety and or paranoia to the drug. Interestingly, frequency of cannabis use was also associated with the pleasurable response (Factor 3) although only marginally (p-value=0.05). Adding number of copies of the LPS haplotype, age of first use and type of cannabis used did not show significance. Effect was independent of disease status (p-value=0.6) and ethnicity (p-value=0.5).

No association was observed when number of copies of the Val risk allele, frequency of cannabis use, age of first use and type of cannabis used were analysed in relation to the 4 Factors.

Table 7.3 Association between psychosis and factors

VARIABLE	CASES	CONTROLS	BETA VALUE	Std. Error	P-VALUE/CORRECTED P-VALUE
Anxiety/Paranoia response	60	53	0.09	0.16	0.31
Inhibitory/depressive response	60	53	0.07	0.16	0.45
Pleasurable response	60	53	0.19	0.15	0.05
Perception abnormalities response	60	53	0.06	0.143	0.4

Table 7.4 Association between number of copies of the Val risk allele at the rs4680 locus and factors

VARIABLE	CASES	CONTROLS	BETA VALUE	Std. Error	P-VALUE
Anxiety/Paranoia response	41	25	-0.09	0.15	0.4
Inhibitory/depressive response	41	25	-0.1	0.13	0.4
Pleasurable response	41	25	0.04	0.13	0.7
Perception abnormalities response	41	25	-0.22	0.09	0.11

Table 7.5 Association between number of copies of the LPS haplotype and factors

VARIABLE	CASES	CONTROLS	BETA VALUE	Std. Error	P-VALUE/CORRECTED P-VALUE
Anxiety/Paranoia response	48	34	-0.12	0.16	0.2
Inhibitory/depressive response	48	34	-0.23	0.14	0.02/0.08
Pleasurable response	48	34	0.18	0.14	0.9
Perception abnormalities response	48	34	0.21	0.12	0.04/0.16

Table 7.6 Association between frequency of cannabis use and factors

VARIABLE	CASES	CONTROLS	BETA VALUE	Std. Error	P-VALUE/CORRECTED P-VALUE
Anxiety/Paranoia response	60	53	0.32	0.16	0.001/0.004
Inhibitory/depressive response	60	53	-0.01	0.17	0.9
Pleasurable response	60	53	0.19	1.16	0.05/0.2
Perception abnormalities response	60	53	0.06	0.15	0.53

7.5 Summary of results

Cannabis experience Questionnaire was administered to psychotic patients and healthy volunteers. Complete data were collected for 113 subjects, 60 patients and 53 controls. 19 items from the questionnaires were taken into consideration for analysis in this chapter, sections 15.14 and 15.15, recording perceived retrospective experiences during and after use of cannabis. Results of factorial analysis showed that the 19 variables could be explained by 4 factors, each referring to a dimension of experience:

- FACTOR 1: Anxiety/paranoia response
- FACTOR 2: Inhibitory/depressive response
- FACTOR 3: Pleasurable response
- FACTOR 4: Perception abnormality response

Further analysis to explore the role of genetic background and a) frequency of cannabis use b) age of first use showed:

- Subjects with no copies or 1 copy of the LPS risk haplotype experienced a more inhibitory/depressive response upon consumption of cannabis. Moreover, subjects with 2 copies of the LPS haplotype seemed to experience more perception abnormalities. This however did not retain significance after Bonferroni correction for multiple testing (adjusted p-value=0.08 and p-value= 0.16 respectively). It is therefore not a true association.
- Subjects using cannabis more than 3 times per week were more likely to respond with anxiety and or paranoia to the drug (adjusted p-value=0.004).
- Subjects using cannabis more than 3 times per week were also more likely to have a pleasurable experience upon cannabis consumption. This did not however retain significance after multiple testing correction (adjusted p-value=0.2).
- Associations observed were not disease group or ethnicity dependent.

No other association was found.

7.6 Conclusions

This chapter explores the relationship between perceived experiences during and after consumption of cannabis and genetic factors, namely number of copies of the LPS haplotype and number of copies of the risk Val allele at rs4680 locus. Furthermore, it attempts to elucidate the role of early start of cannabis use, such as before adolescence, frequency of use and type of cannabis used. The cannabis experiences questionnaire used for this chapter is a revised version of the CEQ created by Barkus et al., in 2006. The questionnaire used in this chapter was elaborated by the Genetics and Psychosis Study at the Institute of Psychiatry and is enriched in

many items. The GAP CEQ, in fact was designed to obtain data on a wide range of cannabis related events. It prompts subjects to answer questions on age of first use, reason behind the first use and the perpetuation of the behaviour, pattern of use and type of self administration, dose/amount used and relation to money spent, information on any other drug used. This provides our study with a realistic, self perceived report on a range of aspects of cannabis use in psychotic subjects and healthy volunteers. Variables analysed in this chapters are from sections 15.14 and 15.15 of the GAP CEQ (Appendix) and refer to experiences during and after consumption. 19 variables were taken into consideration and reduced to 4 factors with a Principal Component Analysis. The 4 factors clearly show the 4 different dimensions of perceived experiences during drug abuse. Factors were independent between each other and measured 1) anxiety/paranoia response 2) inhibitory/depressive response 3) pleasurable response and 4) perception distortion response. As discussed in the results, the KMO test value of more than 0.5 indicated the adequacy of the sample size. It is therefore safe to assume that the GAP CEQ data analysed in this study addresses with competency all domains of experiences upon drug use. Experiences are of particular interest as they help with the understanding of the mechanisms of action of cannabis. Barkus and colleagues, after administering the CEQ and the Schizotypal Personality Questionnaire (SPQ), showed that the higher subjects scored in the SPQ, the more likely to be experience psychotic like experiences during cannabis use and negative effects afterwards (Barkus et al., 2006). Findings in this chapter are not in line with the literature, in that patients seem to be more likely to experience increase in perception distortion experiences. It is well known that cannabis use tends to result in experiences similar to the positive symptoms observed in schizophrenia. Findings also supported by experimental studies on the effects of Δ9-THC (De Souza et al., 2004) (Morrison et al., 2009) (Morrison and Stone, 2011). In this chapter, there was no significant difference in perceived experiences among the group of patients and controls. Subjects using cannabis 3 or more times per week though, were more likely to experience negative symptoms such as anxiety and or paranoia (Factor 1) (adjusted p-value=0.004), but also pleasurable effect (although not statistically significant after Bonferroni correction). Experimental studies showed that CBD can exert anti-psychotic properties and it is able to moderate the psychoactive properties of Δ9-THC (Englund et al., 2013). It is possible that results observed in this study are due to Δ9-THC-CBD ratio content of cannabis consumed by subjects. Subjects that reported a marked increase in paranoia and/or anxiety could be consuming stronger type of cannabis with higher Δ9-THC content, whereas subjects experiencing a pleasurable effect could be consuming cannabis with a higher CBD content.

Di Forti and colleagues (Di Forti et al., 2009) demonstrated that patients were more likely to use cannabis more frequently (3 or more times per week) and to use stronger types of cannabis (Skunk, Sinsemilla). Δ9-THC is found in higher concentration in stronger types of cannabis

(Sinsemilla) and in the flower part of the plant (Skunk). The ratio of the 2 compounds in the plant, gives the strength of the drug and it is usually linked to exacerbation of symptoms in patients and positive like symptoms in controls. Our results however showed that the association between frequency of cannabis use and anxiety/paranoia and/or pleasurable effect were independent of disease status.

The fact that self reported perceived experienced during and after consumption were pooled together in an attempt to catch a better glimpse of the whole cannabis use phenomena may have lead to confused results where the real effect is diluted because of the merging. Barkus et al., measured experiences during and after cannabis use separately to investigate the "amotivational syndrome" or negative after effects and indeed found an association with high schizotypal scoring subjects. (Barkus et al., 2006). It would be interesting to analyse the two parts of the GAP CEQ separately to see whether there is a replication of the findings in literature. This however was not possible because of the sample size and because the number of tests already performed in this chapter. Analysing the same data in too many different ways would have made results more prone to type 1 error.

This chapter also focuses on the question of whether subjects with a particular genetic background experience different responses to cannabis consumption and whether this helps perpetuate drug abuse behaviour. The question of whether genetic variants moderate the effect of cannabis in the brain has been widely discussed in literature with conflicting results. One of the most representative studies on this subject was conducted by Henquet and colleagues. They found subjects with the Val COMT allele to be more likely to experience psychotic like effect after cannabis use, although subjects scored high for psychosis liability (Henquet et al., 2006). Other studies though found no evidence of interaction between polymorphisms within the COMT gene and cannabis use (Munafo et al., 2005) (Costas et al., 2011) (Zammit et al., 2011).

In this chapter I found evidence that subjects with no copies or 1 copy of the LPS, the a priori set risk haplotype, experienced a more inhibitory/depressive response upon consumption of cannabis. Interestingly, results also showed that subjects with 2 copies of the LPS haplotype tend to experience perception abnormalities upon consumption of the drug (p-value=0.04). These associations were not dependent on disease status or ethnicity, but however did not retain significance after Bonferroni correction for multiple testing (adjusted p-value=0.08 and p-value=0.16 respectively). The LPS haplotype contains rs4680 Val allele, so in line with the observations done by Henquet and colleagues; having more copies predisposes subjects to experience more perception abnormalities upon cannabis use, although in this study psychosis liability was not analysed. There are also similarities with the study of Caspi and colleagues that found subjects with the Val allele having a five-fold increase of risk for psychosis if they smoked

cannabis (Caspi et al., 2005). Furthermore, Estrada et al., found that carriers of the Val allele who used cannabis had an earlier onset of psychiatric disorders (Estrada et al., 2010). Finding supported by animal studies (O'Tualthaigh et al. 2012) and by experimental studies on Δ9-THC, the psychoactive component of cannabis. Although results in this study did not retain significance after Bonferroni correction for multiple testing, they can be seen as indication of the involvement of the Val allele in moderating the effect of cannabis and certainly have biologic plausibility. Having more copies of the Val risk allele at the rs4680 locus, in this study, was not associated with any of the experiences or other measures. Age of first use and type of cannabis were not associated with genetic factors or with any of the experiences. As mentioned earlier, LPS haplotype is made of 4 SNPs, namely rs6260, rs4818, rs4633 and contains the Val allele at the rs4680 locus. It has been shown to be associated with greater enzymatic activity (Nackley et al., 2006), and it is therefore argued to be a better measure of the COMT gene enzymatic activity. This could explain the lack of association at the rs4680 locus and the trend towards significance of the LPS haplotype. It can also be explained by lack of statistical power, because of the sample size. As shown in the previous chapters of this thesis, MAF and frequency of haplotypes were very low, and analysed in this chapter, is only a subset of the total sample. Because of low numbers, subjects in this chapter were pooled together independent of ethnicity. MAF and frequency of haplotypes were different in the 2 sets of samples and although combining the samples does not necessarily increase statistical power, this subset of samples is very well characterised with several cannabis measures and co-varing for ethnicity showed no significance.

The novelty of this chapter resides in the fact that it can analyse together very detailed self reported experiences perceived during and after cannabis use, candidate genetic variants and other aspects of cannabis consumption such as frequency, consumption in adolescence and type of cannabis used.

It is however, advisable to collect a bigger sample size, ethnically characterise it and then analyse again cannabis experiences and genetic factors. Results of this chapter therefore, should be interpreted in light of many limitations. Together with the above described limitations, it is important to mention the nature of the data collected. They are all self reported, self perceived experiences described retrospectively, thus prone to error. Furthermore, type of cannabis was dichotomised in strong VS non-strong, according to the supposed content of Δ9-THC, which is the main psycho-active component. Subjects were not asked to bring a specimen of the drug used for analysis or biological specimen tested for drug metabolites (urine, hair, blood). This would have been unlikely to enhance accuracy of the data as reported experiences were based on past experiences with the inclusions of subjects who are not current users but were able to give an account of perceived past experiences. Overall, these finding do not clearly implicate the role of the LPS haplotype or the frequency of cannabis use in giving more anxiety, depression and or

paranoia response to the drug. There is the need of a much larger sample for this. They can however be seen as an indication of involvement of the COMT gene in moderating the effects of cannabis use, partially in line with the literature available.

CHAPTER 8

RESULTS

The (AAT)n microsatellite of the Cannabinoid Receptor 1 gene in First Episode of Psychosis

8.1 Background information

The Human Cannabinoid receptor 1 gene (CNR1) is located at chromosome 6q14-q15 and has a length of 5.9kb. It consists of 2 isoforms (CNR1a and CNR1b) both containing 4 exons, 3 of which were identified by Zhang et al., in 2004. The AATn microsatellite polymorphism is a trinucleotide short-tandem repeat with 9-10 alleles, ranging from 7 to 18 repeats, with their frequencies being different across different populations. It is located 18,086 bp 3' to the exon 4 (Zhang et al., 2004) and has been studied in association with schizophrenia in 6 studies with conflicting results. The first study to investigate the role of the AATn microsatellite was in 2000, by Tsai and colleagues, on 127 Chinese schizophrenic patients and 146 controls. No evidence of association with schizophrenia was found (Tsai et al., 2000). Subsequently, Ujike and colleagues found the AATn microsatellite (MS) to be associated with schizophrenia and especially the hebephrenic subtype in a Japanese population (Ujike et al., 2002), findings partially replicated by Chavarria-Siles et al. in a family based association study of Central Valley and Costa Rica population (Chavarria-Siles et al., 2008). The AATn MS was associated with hebephrenic schizophrenia but not with a wider definition of the disorder (Chavarra-Siles et al., 2008). Martinez-Gras et al., found allele 4 of the AATn MS (10 repeats) to be protective against schizophrenia (Martinez-Gras et al., 2006) in a sample of 113 schizophrenic patients and 111 healthy controls (Martinez-Gras et al., 2006), whereas Siefert and colleagues failed to find any association of the AATn microsatellite and schizophrenia (Seifert et al., 2007). Moreover, Zhang et al., found the AATn MS not to be associated with schizophrenia in a sample of European-American, African-American and Japanese schizophrenic patients and healthy volunteers (Zhang et al., 2004). Difference in allele frequencies across populations was confirmed (Zhang et al., 2004). The AATn MS has also been studied in other disorders including Parkinson's disease (Barrero et al., 2005); Multiple sclerosis (Ramil et al., 2010) (Rossi et al., 2011); Anorexia nervosa (Siegfried et al., 2004) (Muller et al, 2008); Tourette's syndrome (Gadziki et al., 2004), all with conflicting results. Furthermore, many studies have been conducted to analyse the role of the AATn microsatellite in drug addiction. Benyamina and colleagues in a recent meta-analysis of 11 studies, found that the AATn microsatellite within the CNR1 gene showed association with substance abuse in Caucasian population (Benyamina et al, 2011). The three

main areas of investigation of the AATn microsatellite are in schizophrenia, drug abuse and multiple sclerosis. The latest is being increasingly investigated because of the substantial role CNR1 and CNR2 play within the immune system. This chapter will investigate the role of the AATn microsatellite in 2 of the main areas of research: psychosis and cannabis use. Furthermore, it will try to explore its role in the perception of the experience that follows consumption of the drug, based on self reported data.

8.2 Hypothesis under investigation

This chapter will analyse the main effect of the AATn microsatellite on psychosis in the GAP Caucasian and Black populations and in the PICOS Caucasian population. I hypothesise that the AATn microsatellite will have a main direct effect on psychosis with some alleles being over represented in patients.

Furthermore, I will analyse the role of the AATn microsatellite in relation to lifetime cannabis use and depressive/inhibitory perceived experience while consuming cannabis, in order to explore its role.

8.3 AATn Microsatellite genotyping

8.3.1 Custom made primers

Primers used to genotype were computed with Primer3 (Rozen and Skaletsky, 2000; Untergasser et al., 2007) and ordered through Applied Biosystems as follow:

Forward primer: 5' – Fam – AACATGCAGCACACCAACAT – 3'

Reverse primer 5' –GTTTCTTCCTTCTCCCAGCACAATCAT – 3'

DNA was amplified using PCR. The total length of the fragment is of 226 base pairs. Underlined are 19 AAT repeats reported by the online source: UCSC in-silico PCR. The total length of the polymerase chain reaction is of 169 base pairs plus the number of repeats.

AATn:
AACATGCAGCACACCAACATggcacatgtatacatatgtaactaacctgcatgttgtgcacatgtaccctaaaacttaaa
gtataataataataataataataataataataataataataataataataataataataaagattacacctctttcccttaatggcagggttaa
cagaagcagcattttgtgacacATGATTGTGCTGGGAGAAGGGTGAAAG

8.3.2 Sample preparation and Polymerase Chain Reaction

DNA was diluted at 10ng/µl for all samples and stored in 96-well plates at 4°C. All DNA from cases and controls were mixed randomly between the plates to allow for PCR and fragment analysis to be carried out in a blinded fashion. Plates underwent a 1 minute spinning cycle in an ALC multispeed refrigerated centrifuge. 1 microlitre (µl) of each sample was then transferred to two 384-well plates and DNA left to dry overnight.

Primers were lyophilized and prepared with 146µl dd H_2O for the forward primer, and 175µl dd H_2O for the reverse primer. Deoxynucleotide Triphosphate (dNTP) was mixed using 4µl each of dATP, dCTP, dTTP and dGTP and 48µl dd H_2O. PCR was performed in a 5µl reaction volume as follow:

Reagents	**Volume needed for 5µl reaction**
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Gold Buffer	0.5 µl
dNTP	0.5 µl
AmpliTaq Gold DNA polymerase	0.025 µl
$MgCl_2$	0.4 µl
dd H_2O	2.575 µl
DNA	dried

To establish optimum annealing temperature for the primers, a gradient PCR of 50 – 60 °C was performed in a BIO-RAD MJ Peltier Thermal Cycler. Gradient program as follow:

1	Incubate at 95°C for 10 minutes
2	Incubate at 95°C for 10 seconds
3	Gradient from 50 – 60 °C for 10 seconds
4	Incubate at 72°C for 30 seconds
5	Go to line 2 for 30 more times
6	Incubate at 72°C for 5 minutes
7	Incubate at 4°C forever

Samples were then loaded on agarose gel (1.5g UltraPure Agarose, 100 ml of 1xTBE buffer, and 2µl 100 x Gel Red dye) and run at 115 Volts for 40 minutes. Gel was observed under ultraviolet light and compared to a 100 base pair ladder (1.5 µl loaded). Annealing temperature chosen was 61°C, PCR program was therefore set as follow:

1	Incubate at 95°C for 10 minutes
2	Incubate at 95°C for 10 seconds
3	Incubate at 61 °C for 10 seconds
4	Incubate at 72°C for 30 seconds
5	Go to line 2 for 30 more times
6	Incubate at 72°C for 5 minutes
7	Incubate at 4°C forever

8.3.3 Fragment Analysis

Samples were loaded into 384 well plates in a 11.5µl reaction volume as follow:

Reagents	Volume needed for 11.5µl reaction
PCR product (DNA)	1 µl
HiDi Formamide	10 µl
GeneScan 500 ROX size standard	0.5 µl

The reaction was denatured at 95°C for 2 minutes in the BIO-RAD MJ Peltier Thermal Cycler. The plates were then run in an Applied Biosystems 3130xl Genetic analyzer.. Results from fragment analysis were viewed using GeneMapper v4.0 software, and the size of each allele in base pairs was recorded from electropherographs.

Each plate included duplicate samples for quality control. No discrepancies were found in any of the plates.

8.4 Statistical Analysis

In this chapter, the samples analysed are from the GAP Study (both Caucasian and Black population included) and from the PICOS study.

The GAP Study Caucasian population analysed consisted of 174 psychotic patients and 45 non psychotic subjects;

The GAP Study Black population analysed consisted of 113 psychotic patients and 95 non psychotic subjects;

The PICOS Study sample consisted of 347 psychotic patients and 307 non psychotic patients.

The three main groups of samples were tested separately: Caucasian group (GAP); Black African group (GAP); Caucasian Italian group (PICOS) in order to account for ethnic differences.

8.4.1 Statistical tests performed

8.4.1.1 Calculation of main effect on Psychosis

With the three sets of samples, namely the GAP study sample (including Caucasian subjects), the GAP study (including the black population) and the PICOS study sample (consisting of Caucasian population) the following statistical tests were performed:

- Logistic regression test for allelic association analysis between the AATn microsatellite and disease status
  Linear regression test for correlation between the AATn microsatellite and cannabis use
- Power calculation

Exploratory analysis was performed in the GAP Caucasian and Black samples separately:

- Linear regression test for the Gene x Environment analysis (only the GAP Caucasian and Black study sample) to asses correlation between the AATn microsatellite and a) cannabis use b)Factor 2 (depressive/inhibitory response to cannabis use)

8.4.1.2 Allelic association analysis

Statistical analysis carried out to compare allelic distribution between cases and controls using CLUMP software for genetic analysis (Sham and Curtis, 1997). CLUMP is software that uses the Monte Carlo approach to assess significance by performing repeated simulations of contingent tables with the same marginal totals as the one under consideration (Sham and Curtis, 1997). The significance level is therefore unbiased independently of continuity correction or small expected values. The χ^2 value is calculated by summing all cells and dividing the difference between observed and expected value by expected values (Sham and Curtis, 1997). CLUMP generates 3 statistics: T1 with χ^2 from original table; T3 with χ^2 obtained by comparing each column against the rest and T4 with χ^2 calculated from a 2x2 table with clumped alleles to maximise χ^2 value. This is the statistical test considered in this study. The procedure of clumping is obtained by dividing the columns into those with higher than expected values in the first row from those with lower values (Sham and Curtis, 1997). The full command line used to perform analysis was:

CLUMP inputfilename outputfilename

CLUMP produces an outputfile with χ^2 values from each of the 3 statistics together with the number of times such value was reached in the simulation table, the less simulations reached, the more significant the result will be (Sham and Curtis, 1997).

8.4.1.3 Exploratory analysis

Correlation between clinical variables and the copies of the AATn microsatellite was calculated with linear regression using IBM SPSS version 19.

In line with literature I decide to further explore my data by adding to the model

a) Cannabis use

b) Factor 2 (depressive/inhibitory response to cannabis use)

As mentioned in the background information, 2 studies (Ujike et al., 2002) (Chavarria-Siles et al., 2008) found the AATn microsatellite to be associated with hebephrenic schizophrenia.

To diagnose hebephrenic schizophrenia Ujike et al., referred to the ICD-10 whereas Chavarria-Siles et al., used the Lifetime Dimension of Psychosis (LDPS) as the DSM-IV does not include this diagnosis. Hebephrenic schizophrenia is largely characterised by predominance of disorganised and negative symptoms, although this is not formally included in the classification of the disorder by the ICD-10 (Ujike et al., 2002). As noted by Solowij and Michie, in 2007 and by Chavarria-Siles in 2008, the hebephrenic type of schizophrenia closely resembles the cognitive dysfunctions observed in schizophrenic patients abusing cannabis. My sample does not consist of individuals with the diagnosis of hebephrenic schizophrenia but does include patient and control data on cannabis use. Furthermore, individual experiences while and after taking cannabis were measured with the GAP Cannabis Experience Questionnaire, adapted from Barkus et al., 2006. As illustrated in chapter 7, experiences can be grouped in 4 factors, one of which (Factor 2 labelled as depressive/inhibitory response) can resembles some of the features seen in hebephrenic schizophrenia. I therefore decided to run an exploratory analysis to assess better the role of the AATn microsatellite in drug abuse (cannabis) and particularly in subjects that have reported a negative experience while self administering the drug.

Alleles were collapsed into 2 groups, short alleles with less than 12 repeats and long alleles with more than 12 repeats, as reported in previous studies on association of the AATn microsatellite and drug abuse (Coming et al., 1997) (Li et al., 2000). This was performed also in light of the possible role played by the length of the repeats in gene regulation as illustrated by Li and colleagues in 2004.

Individuals were divided into 3 different groups depending on their genotype:

1. Homozygous individuals (genotype of <12repeats,<12repeats)

2. Heterozygous individuals (genotype of <12repeats,>12repeats)

3. Homozygous individuals (genotype of >12repeats, >12repeats)

Association was then assessed using a linear regression test with IBM SPSS version 19.

Factor 2 was entered in the regression models as a dependent variable. The following variables were entered as independent variables:

- Cannabis use: yes VS no (variable modelled as binary trait: 1,0)
- AATn microsatellite genotype (3 groups: homozygous short allele, heterozygous, homozygous long allele)

8.5 Results

8.5.1 Association analysis

In the GAP sample, after fragment analysis, 12 alleles were found at the AATn microsatellite locus, ranging from 5 to 17 repeats, with 6 repeats being absent and 5, 7, 8, 15 repeats being rare and 16, 17 repeats being very rare (Table 8.1).

In the PICOS sample, after fragment analysis, 14 alleles were found at the AATn microsatellite locus, ranging from 3 to 19 repeats, with 8, 17 and 18 repeats being absent. 4, 6 and 19 repeats alleles were rare (Table 8.2).

These finding are in line with literature on the subject, except for the 19 repeats allele which has never been observed before and has been only found in 3 patients of the PICOS study.

As it can be seen from tables 8.1 and 8.2, there are marked allele frequency differences among the 3 samples examined, this is most likely due to ethnic difference among samples.

Table 8.1 Frequency percentage distribution of the AAT microsatellite alleles in the GAP Study Sample (Caucasian and Black populations)

	(AAT)5	(AAT)7	(AAT)8	(AAT)9	(AAT)10	(AAT)11	(AAT)12	(AAT)13	(AAT)14	(AAT)15	(AAT)16	(AAT)17
Caucasian cases	0	1.11%	1.80%	17.10%	29.80%	19%	11.50%	7.80%	10.44%	0.70%	0.37%	0
Caucasian controls	1.47%	0	0	20.50%	33.80%	10.29%	13.20%	7.35%	13.20%	0	0	0
Black cases	0.50%	1.13%	0.50%	8.50%	26.10%	3.90%	14.70%	22.70%	20.45%	0.50%	0	0.50%
Black controls	0	0	0	5.19%	24%	8.40%	15.58%	18.80%	26.60%	1.29%	0	0

Figure 8.1 Graphic representation of the distribution of the alleles of the AATn microsatellite in the GAP Caucasian group

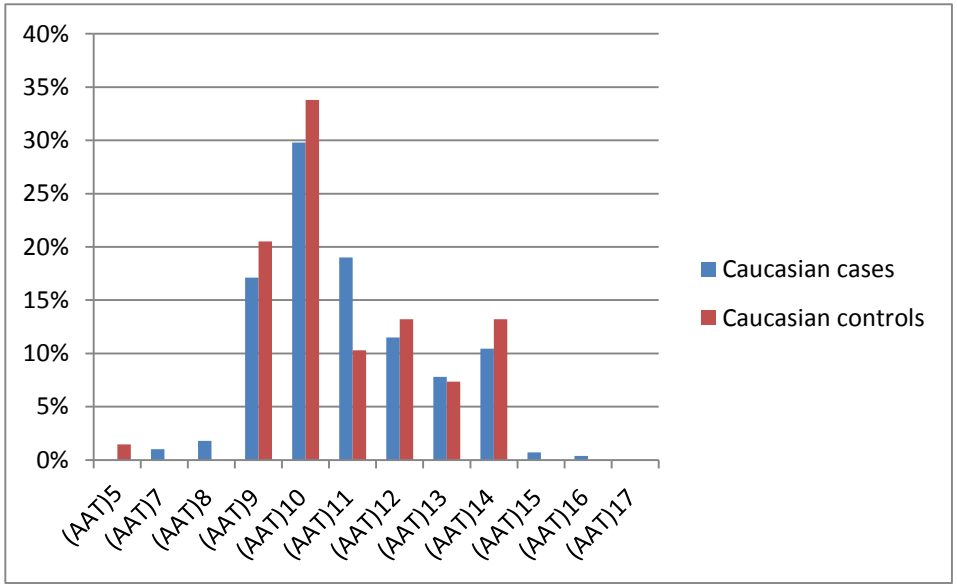


Figure 8.2 Graphic representation of the distribution of the alleles of the AATn microsatellite in the GAP Black group

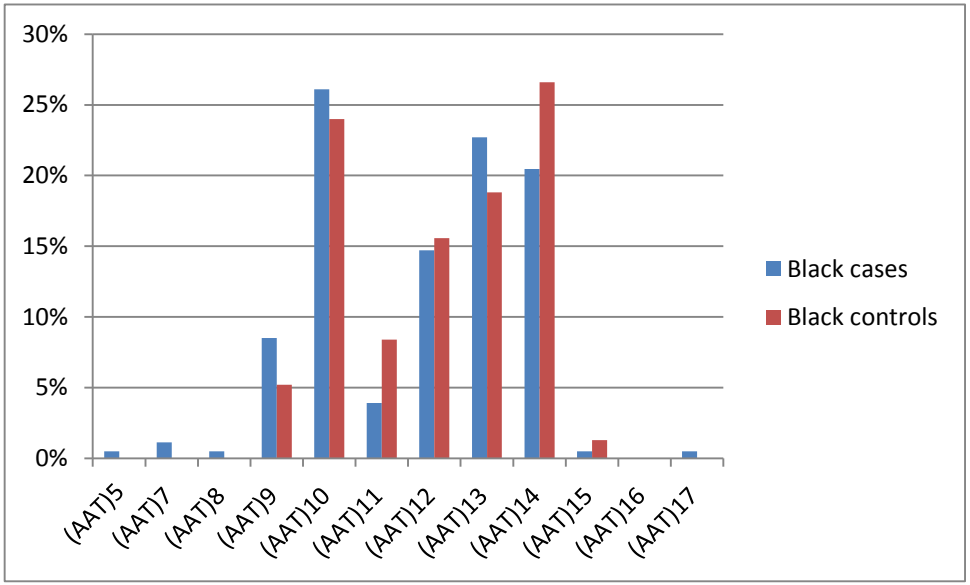
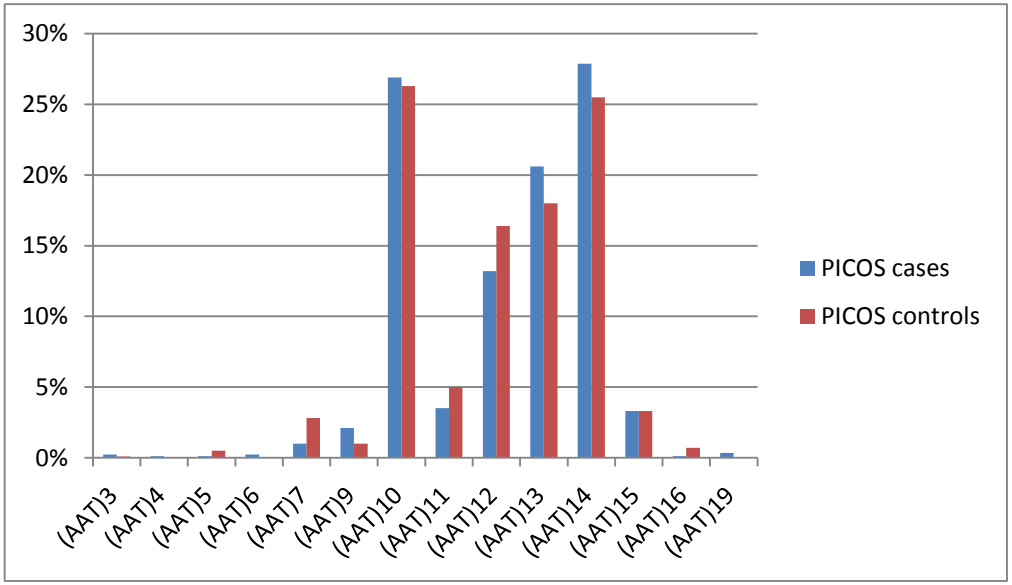


Table 8.2 Frequency percentage distribution of the AAT microsatellite alleles in the PICOS Study Sample

	(AAT)3	(AAT)4	(AAT)5	(AAT)6	(AAT)7	(AAT)9	(AAT)10	(AAT)11	(AAT)12	(AAT)13	(AAT)14	(AAT)15	(AAT)16	(AAT)19
PICOS cases	0.23%	0.11%	0.11%	0.23%	1%	2.10%	26.90%	3.50%	13.20%	20.60%	27.87%	3.30%	0.11%	0.35%
PICOS controls	0.10%	0	0.50%	0	2.80%	1%	26.30%	5%	16.40%	18%	25.50%	3.30%	0.70%	0

Figure 8.3 Graphic representation of the distribution of the alleles of the AATn microsatellite in the PICOS Study sample



Association analysis between the AATn microsatellite and disease status returned no positive association in any of the 3 groups, the AATn MS did not have a significant main effect on psychosis.

GAP Study Caucaisan sample:

Chi-square from 2x2 table clumped to produce maximum (T4) was 3.555573

This was reached 54 times in 100 simulations (empirical p=0.544554)

GAP Study Black sample:

Chi-square from 2x2 table clumped to produce maximum (T4) was 4.300313

This was reached 34 times in 100 simulations (empirical p=0.346535)

PICOS Study sample:

Chi-square from 2x2 table clumped to produce maximum (T4) was 13.148986

This was reached 226 times in 10000 simulations (empirical p=0.242698)

8.5.2 Exploratory analysis

Exploratory analysis was carried out only in the GAP Study sample, in both Caucasian and Black group. There was no cannabis information on the PICOS Study sample.

The alleles of the AATn microsatellite were grouped to form 3 classes of genotypes:

<12 <12

<12 >12

>12 >12

Frequencies of the 3 genotypes in the GAP Caucasian and Black samples can be seen in Table 8.3.

Table 8.3 Genotypes percentage distribution in the GAP Study Sample (Caucasian and Black groups)

	short allele homozygous	heterozygous	long allele homozygous
Caucasian cases	46%	51.70%	11.90%
Caucasian controls	45.40%	39.30%	15%
Black cases	18.60%	43%	38.30%
Black controls	11.50%	52.50%	35.80%

Correlation analysis between the AATn microsatellite and cannabis use did not return positive results. The polymorphism did not seem to have an effect on consumption of cannabis.

When Factor 2 (depressive/inhibitory response) was added to the model, it also showed no significant association.

Results can be seen in Table 8.4

Table 8.4 Association analysis between the AATn microsatellite, lifetime cannabis use and Factor 2

VARIABLE	BETA VALUE	Std. Error	P-VALUE
Lifetime cannabis use	0.19	0.21	0.3
Inhibitory/depressive response Factor 2	0.15	0.16	0.4

8.6 Summary of results

In this chapter I analysed the main effect of the AATn microsatellite within the CNR1 gene on psychosis. The samples analysed were the GAP Study sample Caucasian and Black group and the PICOS sample. After initial association tests, I decided to further explore my data by testing the association of the AATn microsatellite with cannabis use and with a depressive/inhibitory response experienced by individuals during and after cannabis use. This analysis was only run in the GAP Caucasian and Black group as cannabis data were not available in the PICOS sample.

- Monte Carlo test showed no association of the AATn microsatellite and psychosis in any of the 3 samples analysed.
- Exploratory analysis did not show any significant association between the AATn microsatellite and lifetime cannabis use and depressive/inhibitory response.

8.7 Conclusions

Results of this chapter failed to show any significant association between the AATn microsatellite within the CNR1 gene and psychosis or cannabis use. Alleles observed in the 3 samples analysed (the GAP sample Caucasian and Black group and the PICOS sample) are mainly in line with literature. Different studies report different number of alleles, with the most frequent of them being the ones from 11 to 15 repeats. The literature also shows difference in allele frequency across ethnically different populations. The closest match to the Caucasian Italian PICOS population can be found in the study of Rossi and colleagues. In a study on Multiple Sclerosis (MS), they analysed a cohort of 350 MS patients of central-southern Italy origins. Frequencies reported in their study vary significantly with those observed in the PICOS sample. The 10 repeats allele for example seems to be rare (1%) in the MS study but rather frequent in the PICOS sample (26%), on the other hand, the 15 repeats allele seems to be more frequent in the MS study (25.7%) than in the PICOS sample (3%). These differences are most probably explained by methodological differences in genotyping and calling of repeat sizes, to correctly compare sizes in different studies ideally representative samples should be exchanged between centres. Differences in frequency can also be observed between the GAP Study Caucasian and Black groups. The GAP Black group can be compared to the sample analysed by Ballon et al, which consisted of a cohort of African-Caribbean schizophrenic patients and controls with cocaine addiction co-morbidity. Although not as striking as differences between the 2 Italian samples mentioned above, the Afro-Caribbean cohort and the GAP Black group had some differences in allele frequency. These observed discrepancies in number of alleles found and frequency of them can be due to ethnic diversity where, for example, even individuals from the same Country share only partial genetic background. In light of such

differences within samples in this chapter and between samples analysed in other studies, I decided not to merge the Caucasian and the Black GAP populations. Although merging would have increased the sample size, it most definitely would not have increased the power of the analysis.

The most positive results in literature, are from studies that have analysed the role of the AATn microsatellite, in hebephrenic schizophrenia. As mentioned earlier in this chapter, hebephrenic schizophrenia is not currently diagnosed with the DSM-IV, but is included in the ICD-10. This sub-type of schizophrenia is characterised mainly by negative symptoms such as blunted affect or emotion, avolition and asociality as described in Ujike et al., 2002). This is what can be also observed in chronic cannabis abuse. Being a cohort of first episode of psychosis, the GAP study does not have diagnosis of schizophrenia or schizophrenia sub-types available. To explore further the relationship between the AATn microsatellite and cannabis abuse in the GAP sample, I opted to analyse the role of the polymorphism in relation to one of the 4 factors analysed in chapter 7 of this thesis. After factor analysis of 19 components of the GAP Cannabis Experience Questionnaire, a revised form of the CEQ (Barkus et al., 2006), 4 factor were highlighted as the ones that could explain the 4 different dimensions of perceived experiences during and after cannabis consumption. Factor 2 was associated with the depressive/inhibitory response. Individuals scoring higher for factor 2, were among those having a less pleasurable and a more similar experience of chronic cannabis abuse. This type of experience can possibly be compared to the features observed in hebephrenic schizophrenia, with the limitation of being a self reported, retrospective perceived experience while taking a drug. Grouping alleles of the AAT microsatellite have also been reported in literature with nearly all studies reporting analysis carried out with groups of alleles. Li and colleagues pointed out a possible role in gene regulation of the length of the repeats of a given microsatellite (Li et al., 2004). For this reason, many studies now report a division of less than 12 or more than 12 repeats of the AATn microsatellite. A recent meta-analysis, however, reported an association of the AATn microsatellite and drug abuse only in Caucasian and only for 16 repeats and above (Benyamina et al., 2010). This division was not possible in this analysis, because alleles with more than 16 repeats, were very rare. I, therefore, decided to align my analysis with literature and divide the genotypes in 2 groups: short <12 and long >12 alleles. There was no significance reached in any of the analysis.

Results reported in this chapter must be read in light of many limitations. First and foremost, as for all the other experimental chapters of this thesis, the sample size is very small and needs to be increased. Power calculation reported in the Materials and Methods chapter, indicates numbers than need to be much higher in order to achieve reasonable power. This can be considered a limitation for all experimental chapters in this thesis. Numbers in the GAP study are further reduced by the need to divide the sample into different groups according to different ethnic origins. This is very

important as the MAF of the polymorphism analysed in the previous experimental chapter and AATn allele frequency shown in this chapter differ consistently across different populations.

Although there were no convincing results in this chapter, further work on this polymorphism is warranted, also in light of the fact that microsatellites have been shown to be able to influence gene expression (Li et al., 2004).

CHAPTER 9

FINAL CONCLUSIONS

9.1 Summary of findings

9.1.1 The Cannabinoid Receptor 1 Gene

9.1.1.1 Hypotheses under investigation

Chapters 5 and 8 addressed the research question of whether common polymorphisms within the CNR1 gene contribute to the pathophysiology of psychosis. In total I analysed 15 tag SNPs covering the complete CNR1 gene and the AATn microsatellite, close to the 3' end of the gene.

All markers were analysed in the GAP Study sample, Caucasian and Black groups, and in the PICOS Study sample.

I hypothesized that:

- Genetic polymorphisms analysed within the CNR1 gene increase risk for psychosis.
- The multiplicative effect of lifetime cannabis use and rs1049353 increases risk for psychosis.
- The AATn microsatellite has a main direct effect on psychosis with some alleles being over represented in patients.

Exploratory analysis was run to:

- Check for haplotypic association with psychosis. Haplotypes were analysed with a sliding window of 3.
- Understand the role of the AATn microsatellite in association with lifetime cannabis use and depressive/inhibitory perceived experience while consuming cannabis.

9.1.1.2 Findings

Results showed that:

- rs1049353 was associated with psychosis in the GAP Caucasian group (corrected p-value=0.03).
- rs806378 was marginally associated with psychosis in the GAP Caucasian group (corrected p-value=0.05).

- No association of any of the markers analysed and psychosis was found in the PICOS Study sample.
- 4 haplotypes were associated with psychosis in the GAP Caucasian group, there was however the presence of an independent significant signal from rs1049353 (p-value=0.02).
- The ACC haplotype was associated with psychosis in the PICOS study, there was however the presence of an independent significant signal from rs806378 (p-value=0.04).
- No Gene environment interaction was found.
- No association of the AATn microsatellite with psychosis was found.
- No association of the AATn microsatellite with lifetime cannabis use or the depressive/inhibitory response to cannabis consumption was found.

9.1.2 The Catechol-O-Methyltransferase gene

9.1.2.1 Hypotheses under investigation

Chapters 6 and 7 addressed the research question of whether candidate polymorphisms within the COMT gene conferred an increase in risk for psychosis. 7 SNPs namely rs737865, rs6269, rs4633, rs4818, rs165599, rs4680 and rs2075507and the LPS haplotype were analysed. The LPS haplotype (a priori) and rs4680 was also investigated in search for evidence of interaction with cannabis use.

Cannabis use data were collected with a variation of the Cannabis Experience Questionnaire. Variable used in the analysis included:

- Lifetime cannabis use
- Frequency of use
- Age at first use
- Type of cannabis used
- Self reported experiences during and after cannabis use

Experiences during and after cannabis consumption were grouped into 4 factors by Principal Component Analysis. Each of them described a different self reported dimension of symptoms experienced:

- FACTOR 1: Anxiety/paranoia response
- FACTOR 2: Inhibitory/depressive response
- FACTOR 3: Pleasurable response
- FACTOR 4: Perception abnormality response

I therefore analysed the relationship between cannabis use, perceived experiences and the mediating role of genetic factors (LPS haplotype and rs4680).

I hypothesised that:

- The analysed SNPs and the LPS haplotype within the COMT gene have a main effect on psychosis.
- The LPS haplotype moderates the risk of psychosis following use of cannabis.

Exploratory analysis was run to:

- Examine the role of the LPS in moderating the risk of psychosis following frequent VS non-frequent use of cannabis, use in adolescence and type of cannabis use.

In regard to the perceived experiences upon cannabis use I hypothesised that:

- Psychotic subjects will experience more anxiety/paranoia or perception abnormality response
- Healthy subjects will experience more pleasurable response
- Subjects with more copies of the risk Val allele will report more anxiety or hallucinatory response
- People with more copies of the LPS haplotype will report more anxiety or hallucinatory response
- Heavier consumers are those who experience more pleasurable response
- Having more copies of the risk Val allele and using cannabis more often predisposes subjects to experience more anxiety/paranoia and or perception abnormalities
- Having more copies of the risk LPS haplotype and using cannabis more often predisposes subjects to experience more anxiety/paranoia and or perception abnormalities

Rs4680 was the only marker analysed in 3 populations: GAP Caucasian, GAP Black and PICOS Study sample.

All other markers were only analysed in the GAP Caucasian and the GAP Black Study samples.

9.1.2.2 Findings

Results showed:

- No presence of main effect on psychosis at any of the loci nor the haplotypes.
- No effect of the LPS haplotype on cannabis use.
- No evidence of Gene x Environmental interaction.

Analysis of the perceived experiences upon cannabis consumption showed that:

- Subjects with no copies or 1 copy of the LPS risk haplotype experienced a more inhibitory/depressive response upon consumption of cannabis. Moreover, subjects with 2 copies of the LPS haplotype experienced more perception abnormalities. This however did not retain significance after Bonferroni correction for multiple testing (adjusted p-value=0.08 and p-value= 0.16 respectively).
- Subjects using cannabis more than 3 times per week were more likely to respond with anxiety and or paranoia to the drug (adjusted p-value=0.004).
- Subjects using cannabis more than 3 times per week were also more likely to have a pleasurable experience upon cannabis consumption. This did not however retain significance after multiple testing correction (adjusted p-value=0.2).
- Associations observed were not disease group or ethnicity dependent.

9.2 Final conclusions

To analyse the role of the Cannabinoid Receptor 1 gene, I selected 15 SNPs covering the complete length of the gene with an aggressive tag method. A good coverage of the region of interest increases statistical power (Evans and Purcell, 2012), this is what I tried to achieve as only having a small study sample. Samples have been further reduced in size as I decided to analyse the GAP Caucasian and the GAP Black groups separately. The Caucasian sample included Caucasian/European subjects whereas the Black population included Black African, Black Caribbean subjects, but also all individuals that reported one of the parents being either Black African or Black Caribbean. Minor Allele frequency (MAF) was in fact different between the two groups. If The 2 samples were to be analysed together, ethnic difference would have been a confounder. Difference in MAFs though, can also be explained by the small sample size. rs1049353 showed significant association with psychosis in the GAP Caucasian group, but not in the GAP Black group or in the PICOS Study sample. Differences in allele frequency are possibly due to ethnicity and are expected to differ between the GAP Caucasian and Black group. The PICOS Study

sample, however, included individuals of Italian Caucasian origins and was therefore expected to resemble the GAP Caucasian sample, although difference in frequency in these two study samples can still be due to natural ethnic difference between Caucasian populations within Europe. Rs1049353 is the polymorphism that has attracted most attention in research; it is a synonymous SNP located in exon 4 of the CNR1 gene, at 1359 base pairs from the start of the translational site at the codon for Thr 453 (Zhang et al., 2004). The rs1049353 A/G base change had both alleles described as risk alleles by different studies (Hadmani et al., 2008) (De Luis et al., 2012), moreover it has been found to be associated with cocaine dependence (Zuo et al., 2009), major depression (Mitjans et al., 2013), lack of improvement in leptin levels (De Luis et al., 2012) and very recently with schizophrenia, although it did not pass multiple correction (Costa et al., 2013). Other polymorphisms within the CNR1 gene have been reported as interesting in literature. Rs806366 was very recently reported to be associated with schizophrenia (Costa et al., 2013), rs806371 with melancholia (Mitjans et al., 2013), rs806374 with Tardive Diskinesia in schizophrenic patients (Tiwari et al., 2012), rs806378 with weight gain in olanzapine and clozapine treated psychotic patients (Tiwari et al., 2010). Rs806378 showed marginal association with psychosis in this study (p-value=0.05). No other association was found with psychosis or cannabis use.

I also analysed the AATn microsatellite within the CNR1 gene, but failed to replicate any findings. To date six studies have focused on the association between the AAT microsatellite and schizophrenia, two of which reporting significant association with the hebephrenic subtype. Hebephrenic schizophrenia is classified as definite and sustained incongruity or inappropriateness of affect or behaviour which is aimless and or disjointed rather than goal directed (ICD-10). Ujike and colleagues defined it as characterised predominantly by negative symptoms, resembling chronic cannabis use (Ujike et al., 2002) and found it to be associated with the AATn microsatellite; these findings were partially replicated by Chavarria-Siles and colleagues in 2008. Because of the resemblance in symptoms between hebephrenic schizophrenia and chronic cannabis abuse, I decided to analyse the role of the AATn microsatellite in relation to Factor 2 derived from the Cannabis Experience Questionaire, which describes experience of depressive/inhibitory type self reported by subjects upon cannabis use, but found no significant association. The hebephrenic subtype of schizophrenia is not stable over time, the positive results showed by Ujike et al. and Chavarria-Siles et al. could be related to methodology such as multiple testing. The same considerations regarding sample size can be made for the analysis of the AATn microsatellite, for which significant differences in alleles frequency were observed across samples analysed in this study but also with frequencies reported by prior published studies. Discrepancies observed could possibly be due to ethnic difference across samples, but are more likely due to methodological differences in genotyping and calling of repeat sizes or indeed related to inaccurate estimation of frequency.

The second gene analysed in this thesis is the Catechol-O-Methyltransferase (COMT) gene. Markers within the COMT gene have been chosen, because they were already reported to be associated with schizophrenia, in previous studies. Rs4680 is with no doubt the most studied polymorphisms within the COMT gene, in relation to schizophrenia and cannabis use. This type of research increased after it was found to interact with cannabis use in moderating the risk for psychosis by Caspi et al., in 2005. Rs4680 is also a biological plausible candidate as it is located in the coding region of the COMT gene, resulting in a valine/methionine substitution at codon 158 in the MB-COMT isoform (Met158Val). This substitution affects the thermostability of the enzyme resulting in reduction of COMT activity in the Met allele carriers and an increase of function in Val allele carriers (Lachman et al., 1996). Rs4680 was not found to be significantly associated with psychosis or cannabis use in this study. Other markers analysed within the COMT gene, namely rs6269, rs4633 and rs4818 have been chosen as part of three different haplotypes named after their ability to influence sensitivity to pain: Low Pain Sensitivity (LPS) haplotype, Average Pain Sensitivity (APS) haplotype and High Pain Sensitivity (HPS) haplotype. The LPS contains the Val allele of rs4680, the allele considered to be risk and showed higher enzymatic activity (Nackley et al., 2006). The LPS haplotype has been analysed in this study in relation to psychosis, to cannabis use and to the different self reported experiences upon cannabis consumption. No association was found to be statistically significant after Bonferroni Correction. Although not significant after multiple testing correction and therefore not statistically relevant, results showed that subjects with no copies or 1 copy of the LPS, the a priori set risk haplotype, experienced a more inhibitory/depressive response upon consumption of cannabis and that subjects with 2 copies of the LPS haplotype tend to experience perception abnormalities upon consumption of the drug (adjusted p-values=0.08, 0.16 respectively). These associations can however be seen as an indications of possible involvement of the COMT gene in moderating the effect of cannabis. The LPS in fact, contains the Val at risk allele, involved in the degradation of dopamine and therefore a logic biological candidate for moderating the effects of cannabis seen in psychosis. Evidences of the involvement of the Val allele in moderating the effect of cannabis come from several studies (Caspi et al., 2005) (Henquet et al., 2006) (Estrada et al., 2010).

Furthermore, another association that lost significance after Bonferroni correction was between amount of cannabis used and two different dimensions of experiences. Subjects using cannabis three or more times per week were more likely to experience negative symptoms such as anxiety and or paranoia (Factor 1) but also pleasurable effects (Factor 3), association was not dependent on disease status or ethnicity, but not however statistically relevant. If we were instead in the presence of a true statistical positive association, these findings could be the result of subjects using different types of cannabis with different Δ9-THC-CBD ratio content and could therefore be experiencing a different response upon use, independently of whether their illness. For example consuming

cannabis mainly containing Δ9-THC everyday it is not comparable to consuming Hash like substances at the same frequency. This is largely addressed in the GAP group by asking subjects to state which type of cannabis they mainly use, but the terms Skunk or Herbal Cannabis only refer to the part of the plant being consumed, not to the potency of it. We could therefore have data from subjects consuming everyday skunk from a plant of medium potency (Δ9-THC content around 10-12%) and from subjects using instead a very potent strain of the plant (Δ9-THC content around 18-20%) in the same category group, thus generating biases. The same applies for terms like Herbal cannabis or Pollen or "Chocolate Hash", but also "Sinsemilla" which refers to a particular method of cultivation of cannabis sativa but that very often is used as synonymous with Skunk. It has been shown, however that patients are more likely to use cannabis more frequently and of a stronger type (Di Forti et al., 2009). I tried to address this issue of conflicting results by including type of cannabis in the analysis, but no significant association was observed.

These contrasting findings could be due to the small sample size but also to the fact that self reported perceived experienced during and after consumption were pooled together in an attempt to catch a better glimpse of the whole cannabis use phenomena, possibly confounding results.

These difficulties can all be considered limitations that arose because of the naturalistic nature of the GAP study.

All results have to be interpreted in light of many limitations widely discussed in the experimental chapters. First and foremost, this study is underpowered; study samples available are too small to correctly detect any significant association. As mentioned in the power calculation paragraph, to achieve 80% power, sample analysed should be in the range of thousands in the presence of common polymorphisms with a small effect size in a complex disorder. This study lacks the ability to detect main genetic effect on psychosis and genetic x environment interaction, it therefore does not provide enough evidence of the involvement of the CNR1 or the COMT gene in the aetiology of psychosis. Subjects, although analysed in separate groups have been pooled together for the analysis on the cannabis experiences, thus causing ethnic stratification. Furthermore, as mentioned earlier, the GAP Caucasian group includes all individuals of Caucasian European background and the GAP Black group includes all individuals of Black African, Black Caribbean and Black African or Black Caribbean mixed ethnicity. This creates a bias as even within Europeans and within Black African/Caribbean descendants there is ethnic difference that can account for genetic difference. Furthermore, ethnicity is self reported, making data prone to biases. A better approach could be to analyse ancestry informative markers on samples before proceeding to association analysis.

Another possible limitation lays in the fact that cannabis consumption and experiences are reported retrospectively, and can therefore be subjected to many biases, such as higher or lower consumption rate.

Heterogeneity of the study samples in terms of diagnosis can also be considered a limitation. There are several advantages on having a cohort of first episode psychosis, but one of the disadvantages is probably the inclusion in the study sample of several different phenotypes. A first episode cohort may include subjects with any of the psychotic disorders and also with affective disorders and although there has been shown that there is genetic overlap to some extent, such heterogeneity of diagnosis may lead to a dilution of the effect size of the markers analyzed.

On the other hand, finding of an association of rs1049353 and rs806378 within the CNR1 gene and the LPS haplotype within the COMT gene with inhibitory/depressive response to cannabis use (although not significant after Bonferroni correction) has plausible biological relevance. The Endocannabinoid Receptor 1 gene is the main binding site of Δ9-THC which is the main psychoactive component of cannabis. Moreover the COMT gene plays a crucial role in the catabolism of dopamine, which has been implied in the pathophysiology of schizophrenia by many studies.

The CNR1 gene is part of the endocannabinoid system and it is widely expressed in human brain. It is highly expressed in the frontal cortex, the basal ganglia, the hippocampus and the cerebellum. All these regions have been proposed as a locus for the pathophysiology of schizophrenia. The CNR1 gene has mainly been studied in regard to fat metabolism, mental health and drug abuse, also because of its localisation within the human brain.

It has been shown that CNR1 knockout mice show D2 receptor hyperactivation and phenotypically resemble schizophrenia (Fritzsche et al., 2001), possibly due to co-localisation of CNR1 with D2 receptors. Furthermore, CNR1 agonists reduce the rate of firing of neurons in hippocampus (Hampson et al., 2000) and impair long term potentiation and execution of working memory tasks (Hill et al., 2004), also observed in prefrontal cortex (Jentsch et al., 1997). It is not surprising that the role of the CNR1 gene has been investigated mainly in substance abuse (Comings et al., 1997) (Li et al., 2000) (Covault et al 2001) (Ballon et al., 2006) (Hoenicka et al., 2007) (Lopez-Moreno 2010) and schizophrenia with conflicting but interesting results. A very recent study has proposed involvement of the CNR1 AATn microsatellite in working memory performance (Ruiz-Conteras et al., 2013), which can be disrupted in acute and chronic cannabis intoxication. Endocannabinoids could therefore modulate the excitatory and inhibitory inputs to dopaminergic neurons. On the other hand the COMT Val allele (present in the rs4680 polymorphisms and in the LPS haplotype) increases dopamine synthesis in VTA neurons. Carriers of the Val allele present with an increase of function of the COMT activity due to reduced enzymatic thermostability resulting in Met allele carriers having a reduction in COMT activity. It has been shown that Val carriers had 38% reduction of COMT activity in PFC tissue in human post mortem studies, with no change in mRNA

transcription (Chen et al., 2004). This leads to reduced degradation and therefore higher levels of catecholamines in the Met allele carriers.

Concurrent stimulation of cannabinoid and dopaminergic receptors, leads to the formation of a D2-CNR1 protein complex that increases cAMP levels, by coupling to Gs (Mackie et al., 1995). This is in contrast with the behaviour usually observed intracellularly (Luzi et al., 2008). This mechanism could potentially hold the key to the interaction between cannabinoid, dopaminergic receptors and cannabis. Genetic differences among individuals could be responsible for moderating this behaviour.

9.3 Future directions

One of the strength of this study, is that data collected on environment, make the Study Sample very well characterised. Data collected with the Cannabis Experience Questionnaire, for example, realistically reflect the range of experiences upon cannabis consumption. Analysing correlation or interaction of these dimensions with genetic predisposing factors, is very interesting and could contribute to the understanding of the interaction between the genetic basis and drug abuse in schizophrenia. A future direction on this matter, would be to increase the sample size, to obtain more statistical power and to analyse experiences, during and after cannabis consumption in a separate fashion. This would avoid possible confounders discussed above. This is especially true in light of the fact that schizophrenia is now considered a complex disorder for which, at least hundreds of genes of small effect are likely contribute and interact, with many different aspect of the environment. A lesson learnt from the GWA studies which only started to discover statistically significant and potentially pathological polymorphisms when sample sizes were increased significantly.

This study gives a contribution to the field investigating on the aetiology of psychosis. It is, to my knowledge, the only one that systematically analysed polymorphisms covering the entire length of the CNR1 gene, exploring its relationship with cannabis use, in a cohort of first episode of psychosis patients and matched controls. Furthermore, it is one of the very few analysing the role of the AATn microsatellite within the CNR1 gene in psychosis. This study is also the first one to explore the relationship of the LPS haplotype with cannabis use and psychosis and with experiences upon cannabis consumption.

Results cannot considered conclusive as the study is significantly underpowered, they can, however, be considered as an indication of the possible involvement of the CNR1 and the COMT gene in the aetiology of psychosis. By implementing points discussed as future directions, this study will be able to give a substantial contribution to the field.

APPENDIX

THE CANNABIS EXPERIENCE QUESTIONNAIRE

SECTION 15 - Cannabis Experiences Questionnaire

Rater's Initials: ..... **Date of Completion:** ..... **/** ..... **/** .....

Not assessed/missing ☐-66 **Participant refused to answer** ☐-88

Instructions to researcher: *Please tick boxes as appropriate to indicate patient's responses. Please be reminded that some questions allow for more than one response.*

15.1 Have you ever smoked/used cannabis? Yes ☐1 No ☐2 *(go to 15.17)*

15.2 How old were you when you first tried cannabis? ..... years

15.3 Why did you first try cannabis? *(You can tick more than one box)*

a) My friends were using it Yes ☐1 No ☐2

b) My family members were using it Yes ☐1 No ☐2

c) To feel better (to get relief from either physical Yes ☐1 No ☐2

or psychological discomfort)

d) Other *(please explain) (not for data entry)* Yes ☐1 No ☐2

.....

Instructions to researcher: *Please consider as current smokers all participants who report usually/customarily smoking cannabis (incl. patients who have not smoked while inpatient/in prison and patients who report occasional use even if it is once every couple of years etc)*

15.4 Do you currently use cannabis?

a) Yes ☐1 *(please answer b, then go to 15.5)* No ☐2 *(please answer c, then go to 15.6)*

b) If **YES**, why did you continue to use cannabis? *(You can tick more than one box)*

i) I like the effect, it gives me a buzz Yes ☐1 No ☐2

ii) It makes me feel relaxed Yes ☐1 No ☐2

iii) It makes me feel less nervous and anxious Yes ☐1 No ☐2

iv) It makes me feel more sociable Yes ☐1 No ☐2

v) Other (please explain) Yes ☐1 No ☐2

.....

c) If **NO**, at what age did you stop? .....

Please state why you stopped *(not for data entry)*:

.....

15.5 Would you like to stop using cannabis one day?

a) Yes ☐1 No ☐2

b) If yes, please explain:

.....

15.6 Does/did cannabis affect your health in any way?

a) Yes ☐1 No ☐2

b) If yes, please explain *(not for data entry)*:

.....

15.7 Does/did cannabis facilitate social situations?

a) Yes ☐1 No ☐2

15.8 How do/did you mostly use cannabis?

a) I smoke/smoked it in a joint with tobacco ☐1

b) I smoke/smoked it in a joint without tobacco ☐2

c) I smoke/smoked it using a bong ☐3

d) I eat/ate or drink/drank it ☐4

e) Other *(please explain)* ☐5

.....

15.9 How often do/did you use cannabis?

a) Every day ☐1

b) More than once a week ☐2

c) A few times each month ☐3

d) A few times each year ☐4

e) Only once or twice ☐5

15.10 When do/did you mostly use cannabis?

a) At weekends ☐1

b) During the day ☐2

c) During the evening ☐3

d) During the day and evening ☐4

e) Other (please explain) ☐5

.....

15.11 Do you/did you mostly use cannabis:

a) Socially (with friends) ☐1

b) On my own ☐2

15.12 On average how much money per week do/did you usually spend on cannabis?

a) Less than £2.50 ☐1

b) £2.50 - £5 ☐2

c) £6 - £10 ☐3

d) £11 - £15 ☐4

e) £16 - £20 ☐5

f) Above £20

15.13 What type of cannabis do/did you mostly use?

a) Hash (cannabis resin/solid) ☐1

b) Imported herbal cannabis ☐2

c) Home-grown skunk/ Sensimilla ☐3

d) Super skunk ☐4

e) Other *(please state)* ☐5

15.14 How often have you had these experiences while smoking cannabis? *Please rate whether it was agood, bad or neutral experience. If rarely or never, ignore rating (good, bad, neutral) and go to next item.*

a) Fearful

i) Rarely or never ☐1

From time to time ☐2

Sometimes ☐3

More often than not ☐4

Almost always ☐5

ii) Good ☐1

Bad ☐2

Neutral ☐3

b) Feel like going crazy/mad

i) Rarely or never ☐1

From time to time ☐2

Sometimes ☐3

More often than not ☐4

Almost always ☐5

ii) Good ☐1

Bad ☐2

Neutral ☐3

c) Nervy

i) Rarely or never ☐1

From time to time ☐2

Sometimes ☐3

More often than not ☐4

Almost always ☐5

ii) Good ☐1

Bad ☐2

Neutral ☐3

d) Suspicious

i) Rarely or never ☐1

ii) Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

e) Feeling happy

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

f) Full of plans/ideas

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

g) Hearing voices

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

h) Able to understand the world better

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

i) Seeing visions

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

15.15 How often have you had these experiences after the initial effects of cannabis have worn off?

Please rate whether it was a good, bad or neutral experience. If rarely or never, ignore rating (good, bad, neutral) and go to next item

a) Not wanting to do anything

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

b) Being suspicious without reason

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

c) Slowed down thinking

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

d) Difficulty in concentrating

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

e) Not able to think clearly

i) Rarely or never ☐1 **ii)** Good ☐1

From time to time ☐2 Bad ☐2

Sometimes ☐3 Neutral ☐3

More often than not ☐4

Almost always ☐5

15.16 Life Time Cannabis History questionnaire.

Instructions to researcher: *Please hand this section over to participant for completion. Explain to participant how to complete this part by using (a) as an example: If you were smoking cannabis when you were 15, were smoking 2-3 joints per day on average, you usually smoked hash and you only smoked by yourself.*

a) AGE RANGE: 0-16

i)Did you use cannabis between the ages of 0 and 16? Yes ☐1 No ☐2

ii) Frequency

Every day ☐1

More than once a week ☐2

About once a week ☐3

About once/twice a month ☐4

A few times each year ☐5

About once a year ☐6

I have only used cannabis once or twice ☐7

iii) Quantity *(average per day)*

1 joint ☐1

2 or 3 joints ☐2

4 or more joints ☐3

iv) Mostly shared:

Yes ☐1

No ☐2

v) Type

Hash (cannabis resin/solid) ☐1

Imported herbal cannabis ☐2

Skunk/ Sensimilla ☐3

Super skunk ☐4

Other ☐5

vi) Setting of use

Socially (with friends) ☐1

On my own ☐2

Both ☐3

b) AGE RANGE: 17-20

i)Did you use cannabis between the ages of 17 and 20? Yes ☐1 No ☐2

ii) Frequency

Every day ☐1

More than once a week ☐2

About once a week ☐3

About once/twice a month ☐4

A few times each year ☐5

About once a year ☐6

I have only used cannabis once or twice ☐7

iii) Quantity *(average per day)*

1 joint ☐1

2 or 3 joints ☐2

4 or more joints ☐3

iv) Mostly shared:

Yes ☐1

No ☐2

v) Type

Hash (cannabis resin/solid) ☐1

Imported herbal cannabis ☐2

Skunk/ Sensimilla ☐3

Super skunk ☐4

Other ☐5

vi) Setting of use

Socially (with friends) ☐1

On my own ☐2

Both ☐3

c) AGE RANGE:ABOVE THE AGE OF 21

i)Did you use cannabis from the age of 21 onwards? Yes ☐1 No ☐2

ii) Frequency

Every day ☐1

More than once a week ☐2

About once a week ☐3

About once/twice a month ☐4

A few times each year ☐5

About once a year ☐6

I have only used cannabis once or twice ☐7

iii) Quantity *(average per day)*

1 joint ☐1

2 or 3 joints ☐2

4 or more joints ☐3

iv) Mostly shared:

Yes ☐1

No ☐2

v) Type

Hash (cannabis resin/solid) ☐1

Imported herbal cannabis ☐2

Skunk/ Sensimilla ☐3

Super skunk ☐4

Other ☐5

vi) Setting of use

Socially (with friends) ☐1

On my own ☐2

Both ☐

15.17 Instructions to researcher: *Please give participant prompt sheet and then read out following instruction: 'Please have a look at the list I handed to you and indicate which drugs you*

use/have used recreationally in the past. Also please state how often you use/have used it, the age at which you first tried the drug(s) and whether you are a past or current user'. Use a new box for each additional drug. Please include tobacco and alcohol where applicable.

a) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

b) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

c) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

d) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

e) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

f) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

g) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

h) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

i) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

j) DRUG:

.....

i) Frequency

Every d.ay ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

k) DRUG:

.....

i) Frequency

Every day ☐1 A few times each year ☐4

More than once a week ☐2 Only once or twice ☐5

A few times each month ☐3

ii) Age .....

iii) Use

Current ☐1

Past ☐2

iv) When

Day ☐1

Night ☐2

Both day and night ☐3

COMPLEMENTARY TABLES

TABLE C.1: Hardy Weinberg Equilibrium (HWE) Test among non psychotic participants from the GAP Black sample

CHR	Gene	SNP	Position	Location	Minor allele/ Other allele	MAF	Genotypes distribution (N)*	subjects (N)	O(HET)	E(HET)	HWE P-Value
6	CNR1	rs10485171	26963224		C/T	0.41	14/31/26	71	0.44	0.49	0.463
6	CNR1	rs806365	26965783		T/C	0.40	8/36/27	71	0.51	0.46	0.609
6	CNR1	rs806366	26967423		T/C	0.49	14/34/22	70	0.49	0.49	1.000
6	CNR1	rs12189668	26969199		C/C	0.06	2\6\65	73	0.08	0.13	0.028
6	CNR1	rs1049353	26973469		A/G	0.25	3/24/48	75	0.32	0.32	1.000
6	CNR1	rs806369	26976012		T/C	0.23	5/16/49	70	0.23	0.30	0.048
6	CNR1	rs806371	26976197		G/T	0.17	4/23/47	74	0.31	0.33	0.723
6	CNR1	rs806374	26977154		C/T	0.37	10/35/29	74	0.47	0.47	1.000
6	CNR1	rs12195101	26977659		G/T	0.04	0/7/67	74	0.09	0.09	1.000
6	CNR1	rs806375	26978355		T/A	0.50	21/31/19	71	0.44	0.50	0.341
6	CNR1	rs806377	26978557		C/T	0.49	21/37/16	74	0.50	0.50	1.000
6	CNR1	rs806378	26979385		T/C	0.30	8/28/39	75	0.37	0.41	0.406
6	CNR1	rs2023239	26980316		C/T	0.22	9/23/40	72	0.32	0.41	0.081
6	CNR1	rs1535255	26981042		G/T	0.17	3/25/44	72	0.35	0.34	1.000
6	CNR1	rs6454672	26981404		C/T	0.14	3/17/51	71	0.24	0.27	0.371

MAF= Minor allele frequency

O(HET)= Observed heterozygosity

E(HET)= Expected heterozygosity

*number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

TABLE C.2: Hardy Weinberg Equilibrium (HWE) Test among non psychotic participants from the GAP Caucasian sample

CHR	Gene	SNP	Position	Minor allele/ Other allele	MAF	Genotypes distribution (N)*	subjects (N)	O(HET)	E(HET)	HWE P-Value
6	CNR1	rs10485171	89261282	C/T	0.44	8/19/8	35	0.54	0.50	0.742
6	CNR1	rs806365	89263841	T/C	0.32	26/67/42	32	0.38	0.45	0.431
6	CNR1	rs806366	89265481	C/T	0.29	6/15/14	35	0.43	0.47	0.720
6	CNR1	rs12189668	89267257	C/T	0.02	0/3/32	35	0.09	0.08	1.000
6	CNR1	rs1049353	89271527	A/G	0.06	2/11/123	35	0.23	0.24	0.526
6	CNR1	rs806369	89274070	T/C	0.12	5/39/95	35	0.23	0.28	0.237
6	CNR1	rs806371	89274255	G/T	0.28	8/34/91	32	0.38	0.34	1.000
6	CNR1	rs806374	89275212	C/T	0.42	5/47/83	31	0.32	0.46	0.119
6	CNR1	rs12195101	89275717	G/T	0.04	19/59/56	35	0.06	0.11	0.086
6	CNR1	rs806375	89276413	A/T	0.45	7/15/12	34	0.44	0.49	0.725
6	CNR1	rs806377	89276615	T/C	0.36	6/15/13	34	0.44	0.48	0.722
6	CNR1	rs806378	89277443	T/C	0.15	1/16/18	35	0.46	0.38	0.395
6	CNR1	rs2023239	89278374	C/T	0.36	10/57/71	35	0.29	0.45	0.055
6	CNR1	rs1535255	89279100	G/T	0.30	2/13/20	35	0.37	0.37	1.000
6	CNR1	rs6454672	89279462	C/T	0.29	1/14/20	35	0.40	0.35	0.651

MAF= Minor allele frequency

O(HET)= Observed heterozygosity

E(HET)= Expected heterozygosity

*number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

TABLE C.3: Hardy Weinberg Equilibrium (HWE) Test among non psychotic participants from the PICOS Study sample

CHR	Gene	SNP	Position	Minor allele/ Other allele	MAF	Genotypes distribution (N)*	subjects (N)	O(HET)	E(HET)	HWE P-Value
6	CNR1	rs10485171	89261282	C/T	0.38	68/257/180	505	0.51	0.48	0.134
6	CNR1	rs806365	89263841	T/C	0.49	121/260/129	510	0.51	0.50	0.723
6	CNR1	rs806366	89265481	T/C	0.46	106/239/159	504	0.47	0.49	0.368
6	CNR1	rs12189668	89267257	C/T	0.00	0/0/510	510	0.00	0.00	1.000
6	CNR1	rs1049353	89271527	A/G	0.23	24/175/303	502	0.35	0.35	0.898
6	CNR1	rs806369	89274070	T/C	0.35	60/233/216	509	0.46	0.45	0.845
6	CNR1	rs806371	89274255	G/T	0.15	9/115/380	504	0.23	0.23	0.847
6	CNR1	rs806374	89275212	C/T	0.33	46/242/219	507	0.48	0.44	0.087
6	CNR1	rs12195101	89275717	G/T	0.00	0/2/512	514	0.00	0.00	1.000
6	CNR1	rs806375	89276413	T/A	0.42	86/261/154	501	0.52	0.49	0.202
6	CNR1	rs806377	89276615	C/T	0.50	123/259/120	502	0.52	0.50	0.532
6	CNR1	rs806378	89277443	T/C	0.26	36/212/259	507	0.42	0.40	0.442
6	CNR1	rs2023239	89278374	C/T	0.17	13/143/354	510	0.28	0.28	0.873
6	CNR1	rs1535255	89279100	G/T	0.17	13/141/350	504	0.28	0.28	0.873
6	CNR1	rs6454672	89279462	C/T	0.12	5/106/400	511	0.21	0.20	0.660

MAF= Minor allele frequency

O(HET)= Observed heterozygosity

E(HET)= Expected heterozygosity

*number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

TABLE C.4: Association between genotype variation at each locus within CNR1 gene and psychosis in the GAP Caucasian sample

Gene	SNP	Minor Allele/Other allele	Genotypes distribution psychotic patients [(a)]	Psychotic subjects(N)[(b)]	Genotypes distribution non-psychotic [(a)]	non-psychotic subjects (N)[(b)]	CHISQ (DF=2)*	P-value
CNR1	rs10485171	C/T	26/79/48	153	8/19/8	35	1.27	0.529
CNR1	rs806365	T/C	16/67/74	157	5\12\15	32	0.88	0.645
CNR1	rs806366	C/T	14/54/84	152	6/15/14	35	3.35	0.187
CNR1	rs1049353	A/G	1/12/143	156	1\8\26	35	NA	NA
CNR1	rs806371	G/T	21/48/88	157	1\12\19	32	NA	NA
CNR1	rs806374	C/T	30/76/53	159	6\10\15	31	3.03	0.220
CNR1	rs12195101	G/T	3/7/147	157	1\2\32	35	NA	NA
CNR1	rs806375	A/T	28/84/43	155	7/15/12	34	1.18	0.554
CNR1	rs806377	T/C	12/86/58	156	6/15/13	34	3.57	0.168
CNR1	rs806378	T/C	2/34/119	155	1/16/18	35	NA	NA
CNR1	rs2023239	C/T	27/59/71	157	7\10\18	25	1.01	0.603
CNR1	rs1535255	G/T	15/71/73	159	2/13/20	35	NA	NA
CNR1	rs6454672	C/T	16/62/76	154	1/14/20	35	NA	NA

(a)= number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

(b)= number of participants (Psychotic and non psychotic) for genotype groups at each locus

* Degrees of freedom=2

TABLE C.5: Association between genotype variation at each locus within CNR1 gene and psychosis in the GAP Black sample

Gene	SNP	Minor Allele/Other allele	Genotypes distribution psychotic patients [(a)]	Psychotic subjects(N)[(b)]	Genotypes distribution non-psychotic [(a)]	non-psychotic subjects (N)[(b)]	CHISQ (DF=2)*	P-value
CNR1	rs10485171	C/T	19/40/38	97	14/31/26	71	0.13	0.938
CNR1	rs806365	T/C	16/51/29	96	8/36/27	71	1.62	0.445
CNR1	rs806366	T/C	24/54/19	97	14/34/22	70	3.11	0.211
CNR1	rs1049353	A/G	8/40/51	99	3/24/48	75	NA	NA
CNR1	rs806371	G/T	3/22/73	98	4/23/47	74	NA	NA
CNR1	rs806374	C/T	17/37/42	96	10/35/29	74	1.43	0.490
CNR1	rs12195101	G/T	2\3\95	100	0/7/67	74	NA	NA
CNR1	rs806375	T/A	27/39/30	96	21/31/19	71	0.40	0.819
CNR1	rs806377	C/T	24/43/32	99	21/37/16	74	2.42	0.298
CNR1	rs806378	T/C	10/39/47	96	8/28/39	75	0.20	0.907
CNR1	rs2023239	C/T	6/23/70	99	9/23/40	72	4.63	0.099
CNR1	rs1535255	G/T	2/25/74	101	3/25/44	72	NA	NA
CNR1	rs6454672	C/T	3/18/78	99	3/17/51	71	NA	NA

(a)= number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

(b)= number of participants (Psychotic and non psychotic) for genotype groups at each locus

* Degrees of freedom=2

TABLE C.6: Association between genotype variation at each locus within CNR1 gene and psychosis in the PICOS Study

Gene	SNP	Minor Allele/Other allele	Genotypes distribution psychotic patients [(a)]	Psychotic subjects(N)[(b)]	Genotypes distribution non-psychotic [(a)]	non-psychotic subjects (N)[(b)]	CHISQ (DF=2)*	P-value
CNR1	rs10485171	C/T	39/122/105	266	68/257/180	505	1.77	0.413
CNR1	rs806365	T/C	59/141/73	273	121/260/129	510	0.51	0.777
CNR1	rs806366	T/C	60/135/68	263	106/239/159	504	2.69	0.261
CNR1	rs1049353	A/G	19/86/162	267	24/175/303	502	2.06	0.356
CNR1	rs806371	G/T	5/83/187	275	9/115/380	504	5.14	0.077
CNR1	rs806374	C/T	27/127/111	265	46/242/219	507	0.30	0.861
CNR1	rs12195101	G/T	0/0/270	270	0/2/512	514	NA	NA
CNR1	rs806375	T/A	35/132/97	264	86/261/154	501	3.72	0.156
CNR1	rs806377	C/T	62/133/71	266	123/259/120	502	0.73	0.693
CNR1	rs806378	T/C	10/104/155	269	36/212/259	507	5.23	0.073
CNR1	rs2023239	C/T	8/83/177	268	13/143/354	510	0.94	0.627
CNR1	rs1535255	G/T	9/79/179	267	13/141/350	504	0.69	0.709
CNR1	rs6454672	C/T	5/64/202	271	5/106/400	511	2.03	0.362

(a)= number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

(b)= number of participants (Psychotic and non psychotic) for genotype groups at each locus

* Degrees of freedom=2

TABLE C.7: Hardy Weinberg Equilibrium (HWE) Test among non psychotic participants from the GAP Caucasian sample

CHR	Gene	SNP	Minor allele/ Other allele	MAF	Genotypes distribution (N)*	subjects (N)	O(HET)	E(HET)	HWE P-Value
22	COMT	rs737865	G/A	0.15	4/42/88	43	0.19	0.21	0.446
22	COMT	rs6269	G/A	0.36	4/16/21	41	0.39	0.41	0.712
22	COMT	rs4633	T/C	0.36	8/19/16	43	0.44	0.48	0.544
22	COMT	rs4818	G/C	0.19	0/15/26	41	0.37	0.30	0.312
22	COMT	rs165599	A/G	0.39	10/13/18	41	0.32	0.48	0.048
22	COMT	rs4680	A/G	0.34	5/14/13	32	0.44	0.47	0.714
22	COMT	rs2075507	G/A	0.35	4/17/12	33	0.52	0.47	0.722

MAF= Minor allele frequency

O(HET)= Observed heterozygosity

E(HET)= Expected heterozygosity

*number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

TABLE C.8 Hardy Weinberg Equilibrium (HWE) Test among non psychotic participants from the GAP Black sample

CHR	Gene	SNP	Minor allele/ Other allele	MAF	Genotypes distribution (N)*	subjects (N)	O(HET)	E(HET)	HWE P-Value
22	COMT	rs737865	G/A	0.2912	4/42/43	89	0.4719	0.404	0.1867
22	COMT	rs6269	G/A	0.4093	19/37/32	88	0.4205	0.4891	0.1945
22	COMT	rs4633	T/C	0.4681	22/39/30	91	0.4286	0.4961	0.207
22	COMT	rs4818	G/C	0.3813	15/40/34	89	0.4494	0.4772	0.6566
22	COMT	rs165599	G/A	0.335	14/36/44	94	0.383	0.4491	0.1689
22	COMT	rs4680	A/G	0.4848	15/36/21	72	0.5	0.4965	1
22	COMT	rs2075507	G/A	0.37	11/25/35	71	0.3521	0.4429	0.1057

MAF= Minor allele frequency

O(HET)= Observed heterozygosity

E(HET)= Expected heterozygosity

*number of participants for genotype groups at each locus presented as homozygous for the minor allele, heterozygous and homozygous for the major allele

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